UNIVERSIDADE DE LISBOA
Faculdade de Medicina Veterinária

Feeding strategies to improve fat partitioning and meat quality in pigs

Marta Sofia Morgado dos Santos Madeira

TESE DE DOUTORAMENTO EM CIÊNCIAS VETERINÁRIAS

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Aos meus pais, Irene e Manuel

Às minhas sobrinhas, Constança e Salomé

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ABSTRACT

Feeding strategies to improve fat partitioning and meat quality in pigs

Modern commercial pig breeds usually produce very lean meat with poor eating quality. Thus, strategies to increase intramuscular fat (IMF) content and consequently to improve sensorial meat quality are needed. In view of this, our investigation explores the potential of feeding strategies based on reduction of dietary protein to improve intramuscular fat and carcass fat partitioning in pigs and to study the associated genetic mechanisms. In a first experiment, three diets differing in dietary protein and lysine levels (control diet with 17.5% crude protein, reduced protein diet – RPD with 13% of crude protein, and reduced protein diet adjusted with lysine – RPDL) were applied to two genetic divergent pig genotypes (30 Alentejano purebred, fatty and 30 commercial crossbred pigs, lean). In a second experiment, six diets differing in dietary protein (16 *vs*. 13% of crude protein) and arginine and/or leucine supplementation were applied on 54 commercial crossbred pigs. The effects of these diets on growth performance, carcass traits, IMF content, meat quality, subcutaneous adipose tissue (SAT) deposition, fatty acid composition and mRNA levels of genes controlling lipid metabolism were evaluated. The results from the first experiment showed that RPD increase IMF but decrease the productive performance in commercial crossbred pigs but not on Alentejano pigs, suggesting that lysine restriction mediates the effect of RPD on muscular lipid deposition. The effect of RPD on IMF of crossbred pigs was accompanied by an increased stearoyl-CoA desaturase (SCD) and *PPARγ* mRNA levels. The backfat thickness did not change with RPD, but total fatty acid content increased in both genotypes. In addition, this result reflects a tendency for higher sensory scores in crossbred pigs. The Alentejano pigs showed higher sensory scores than crossbred pigs, although RPD increased the juiciness of crossbred pigs. In the second experiment, reduced protein diet increased both IMF and backfat thickness. However, the observed increase in IMF was also accompanied by increased *SCD* expression but did not improved meat sensory traits. Neither arginine nor leucine dietary supplementation increased IMF content. Nevertheless, arginine introduced an off-flavour of meat. The only relevant effect of arginine was increasing the fat content of adipose tissue (and consistent up-regulation of fatty acid synthase (*FASN*) and *SCD* genes) and a slightly decrease in 20:5*n-3* and of total *n-3* PUFA in muscle. Leucine supplementation resulted on up-regulation of muscle *SCD* and *FASN* reflected in higher 18:1*c*9 and 16:0 proportions and lower PUFA proportions. The results suggested that fat partitioning can be modulated and pork quality improved with reduced protein diets without lysine adjustment.

Keywords: pigs; reduced protein diets; lysine; arginine; leucine; intramuscular fat; pork quality; fatty acid composition; lipid metabolism.

RESUMO

Estratégias alimentares para melhorar a partição da gordura corporal e a qualidade da carne em suínos

As raças modernas de suínos comerciais normalmente produzem carne muito magra com baixa qualidade sensorial. Deste modo, são necessárias estratégias para aumentar o teor gordura intramuscular (GIM) e consequentemente melhorar a qualidade sensorial da carne. Neste sentido, a nossa investigação explora o potencial de estratégias alimentares baseadas na redução de proteína na dieta para melhorar a GIM e a partição da gordura na carcaça em suínos e também estudar os mecanismos genéticos associados. No primeiro ensaio experimental, três dietas com níveis de proteína e lisina diferentes (dieta controlo, com 17.5% de proteína bruta, dieta reduzida em proteína – RPD com 13% de proteína bruta e uma dieta reduzida em proteína ajustada com lisina – RPDL) foram aplicadas a suínos de dois genótipos diferentes (30 Alentejanos puros e 30 comerciais cruzados). Num segundo ensaio, seis dietas com níveis de proteína diferentes (16 *vs*. 13%) e suplementação de arginina e/ou leucina foram aplicadas a 54 suínos comerciais cruzados. Foram avaliados os efeitos destas dietas na performance de crescimento, nas características da carcaça, no teor de GIM, na qualidade da carne, na deposição do tecido adiposo subcutâneo, na composição em ácidos gordos e nos níveis de mRNA dos genes que controlam o metabolismo lipídico. Os resultados do primeiro ensaio mostraram que a RPD aumentou a GIM mas diminuiu a performance produtiva nos suínos comerciais cruzados, mas não nos Alentejanos, o que sugere que a restrição em lisina medeia o efeito da RPD na deposição lipídica no músculo. O efeito da RPD na GIM nos suínos cruzados foi acompanhado pelo aumento dos níveis de mRNA da delta 9 desaturase (*SCD*) e *PPARγ*. A espessura do toucinho não alterou com a RPD, mas aumentaram os ácidos gordos totais nos dois genótipos. Além disso, este resultado reflecte uma tendência para maiores scores sensoriais nos suínos cruzados. Os suínos Alentejanos mostraram maiores scores sensoriais do que os suínos cruzados, apesar da dieta RPD ter aumentado a suculência nos suínos cruzados. No segundo ensaio experimental, a dieta reduzida em proteína aumentou a GIM e a espessura do toucinho. Contudo, o aumento da GIM foi também acompanhado com o aumento da expressão da *SCD*, mas não melhorou as características sensoriais da carne. Nem a suplementação da dieta com arginina nem com leucina aumentaram o teor de GIM. Porém, a arginina introduziu off-flavor na carne. O único efeito relevante da arginina foi o aumento do teor de gordura no tecido adiposo subcutâneo (consistente com o aumento da expressão dos genes da sintese de ácidos gordos (*FASN* e *SCD*) e um pequeno decréscimo do 20:5*n-3* e do total de *n-3* PUFA no músculo. A suplementação com leucina resultou no aumento de expressão da *SCD* e *FASN* no músculo, reflectida num aumento das proporções de 18:1*c*9 e 16:0 e da diminuição dos teores de PUFA. Os resultados sugerem que a partição da gordura pode ser

modulada e a qualidade sensorial da carne de suíno melhorada com dietas com teor de proteína reduzido sem ajustamento da lisina.

Palavras-chave: suínos; dietas reduzidas em proteína; lisina; arginina; leucina; gordura intramuscular; qualidade da carne; composição em ácidos gordos; metabolismo lipídico.

LIST OF PUBLICATIONS

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

a*	Redness (colour dimension)
ACACA	Acetyl-CoA carboxylase
ADFI	Average daily feed intake
ADG	Average daily gain
Ala	Alanine
AOAC	Association of Official Analytical Chemists
Arg	Arginine
Asn	Asparagine
Asp	Aspartatic acid
ATP	Adenosine triphosphate
b*	Yellowness (colour dimension)
BHT	Butylated Hydroxytoluene
CEBPα	CCAAT/enhancer binding protein alpha
CIISA	Centro de Investigação Interdisciplinar em Sanidade Animal
CLA	Conjugated linoleic acid
cm	Centimetre
CPT-1B	Carnitine palmitoyltranferase 1
ºC	Degree Celsius
CoA	Coenzyme A
CRAT	Carnitine O-acetyltransferase
d	Day
DAD	Diode array detector
DFD	Dry, firm and dark
DHA	Docosahexaenoic acid (22:6*n-3*)
DM	Dry Matter
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DPA	Docosapentaenoic acid (22:5*n-3*)
EPA	Eicosapentaenoic acid (20:5*n-3*)
FABP4	Fatty acid binding protein 4
FADS1	Fatty acid desaturase 1
FADS2	Fatty acid desaturase 2
FAME	Fatty acid methyl esters
FAO	Food and Agriculture Organization
FASN	Fatty acid synthase
FID	Flame ionization detector

g	Gram
G:F	Gain:feed
GC	Gas chromatography
Gln	Glutamine
Glu	Glutamic acid
GLUT4	Glucose transporter type 4
Gly	Glicine
h	Hour
HCl	Hydrochloric Acid
HCW	Hot carcass weight
His	Histidine
HPLC	High-performance liquid chromatography
i.d.	Inner diameter
INIAV	Instituto Nacional de Investigação Agrária e Veterinária
IMF	Intramuscular fat
Ile	Isoleucine
kcal	Kilocalorie
Kg	Kilogram
L*	Lightness (colour dimension)
LC-PUFA	Long-chain polyunsaturated fatty acid
Leu	Leucine
LPL	Lipoprotein lipase
LSMEANS	Least squares means
Lisine	Lys
M	Molar
m	metre
MDA	Malonaldehyde
Met	Methionine
mg	Milligram
min	Minute
mL	Milliliter
MLXIPL	Interacting protein-like
Mm	Milimolar
mm	Milimetre
MUFA	Monounsaturated fatty acid
MUFA/PUFA	Monounsaturated fatty acid/saturated fatty acid ratio
NEFA	Non esterified fatty acid
n-3	Omega 3

n-6	Omega 6
n-6/*n-3*	Total omega 3 fatty acids/total omega 6 fatty acids ratio
P	Probability
PCR	Polymerase chain reaction
pH	negative decimal logarithm of the hydrogen ion activity in a solution
Phe	Phenylalanine
pH_{ult}	ultimate potential of Hydrogen
PPARα	Peroxisome proliferator-activated receptor alpha
PPARγ	Peroxisome proliferator-activated receptor gamma
Pro	Proline
PSE	Pale, Soft and Exudative
PUFA	Polyunsaturated Fatty Acids
rcf	relative centrifugal force (g)
RNA	Ribonucleic acid
RPD	Reduced protein diet
rpm	rotation per minute
RT-PCR	Reverse transcrptase – polymerase chain reaction
SAS	Statistical analysis system
SAT	Subcutaneous adipose tissue
SCD	Steroyl-CoA desaturase
SEM	Standard error of mean
Ser	Serine
SFA	Starurated Fatty Acid
SREBP1	Sterol regulatory element binding protein 1
Tau	Tau
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
UIPA	Unidade de Investigação em Produção Animal
USDA	United States Department of Agriculture
UV	Ultraviolet
v/v	Volume per volume
Val	Valine
VLDL	Very low density lipoproteins
WBSF	Warner-Bratzler shear force

INTRODUCTION

Pork is the most widely consumed meat in Europe. Meat is a major food source of protein and fat. It contains about 20% of protein that provide all essential amino acids and various micronutrients for human diet. Consumption of red meat, including pork and processed pork meat, has been associated to increase the risk of colon cancer, cardiovascular disease and higher cholesterol levels (Ferguson, 2010). However, fat content in meat is a major factor that contributes to these diseases. Consequently, fat content and fatty acid composition in meat has received considerable attention in view of their implications for meat quality characteristics and human health (Wood *et al.*, 2008). In commercial crossbred pigs, the genetic selection has been directed to reduction of carcass fatness resulting also in very low intramuscular fat (IMF) content, which affected the eating quality of meat. It is well established that acceptable pork eating quality requires a minimum IMF of 2.5%. Reduction in IMF below 2% negatively affects the palatability, juiciness and tenderness of pork (De Vol *et al.*, 1988; Eikelenboom & Hoving-Bolink, 1994a). For that reason, pig industry and consumers prefers pork with high amounts of IMF but without an increase in subcutaneous fat.

The amount of IMF is regulated by a combination of factors, such as breed and diet. It also varies between different muscle types. In recent years, there has been a development of nutritional strategies to enhance fat accumulation in muscle without a significant effect on subcutaneous adipose tissue (SAT). Some of these strategies include the use of reduced protein diets (RPDs) (Doran *et al*. 2006), low lysine levels (D'Souza, Pethick, Dunshea, Pluske & Mullan, 2008), high arginine levels (Ma *et al.*, 2010) and high leucine levels (Cisneros, Ellis, Baker, Easter & McKeith, 1996; Hyun, Ellis, McKeith & Baker, 2003 and Hyun *et al.*, 2007) in diets. However, the mechanisms associated to these strategies are not clear. For the RPDs, one possible explanation could be the fact that lower dietary protein levels stimulate expression of muscle lipogenic enzymes, and consequently increase *de novo* fatty acid synthesis. RPD activates stearoyl-CoA (SCD) protein expression that is one of the key lipogenic enzymes, which catalyses the cellular biosynthesis of monounsaturated fatty acids (MUFA) and increases SCD activity in pig muscle but not in SAT (Doran *et al.*, 2006). Also, low lysine diet increase SCD transcriptional rate in pig muscles (Da Costa *et al.*, 2004). Nevertheless, knowlege about the effects of these strategies on the expression of genes encoding the enzymes in lipid metabolism is scarce.

Overall, this work aims to explore feeding strategies to improve fat partitioning with an increase of IMF, meat quality traits of pork without compromising productive performance (growth and carcass characteristics). In addition, the effects of these feeding strategies on genetic and metabolic mechanisms involved in lipid metabolism are investigated.

This thesis is divided in six chapters. The chapter 1, entituled “Scientific background and objectives”, revises our current knowledge on the swine sector, globally and in the Portuguese market. The production systems and swine breeds more used are characterized and pig nutrition is reviewed. Then, pork quality was reviewed, with special attention to meat physical traits and meat lipids (IMF and fatty acid composition). Finally, nutritional regulation of lipid metabolism (the key enzymes and transcription factors) to improve pork quality was reviewed. At the end of this chapter, the objectives of this work are described. Chapters 2, 3, 4 and 5 are organized in papers based on scientific manuscripts already published or submitted to publication in international peer reviewed journals.

The effects of RPDs with or without lysine addition on growth performance and carcass composition of pigs, meat quality traits and sensory traits of pork are presented in chapter 2. Chapter 3 describes the fatty acid composition and gene expression of genes involved in lipid metabolism of muscle and subcutaneous fat in pigs fed RPDs with or without lysine addition. Chapter 4 explores the effects of RPDs with arginine and/or leucine supplementation on growth performance, carcass composition, meat quality traits, free amino acid composition and sensory traits of pork. The fatty acid composition and gene expression of genes involved in lipid metabolism of muscle and subcutaneous fat in pigs fed these diets are presented in chapter 5.

Finally, chapter 6 integrates the results obtained in each of the four previous chapters, and provides an overall discussion, conclusions, implications and futures perspectives.

Chapter 1

SCIENTIFIC BACKGROUND AND OBJECTIVES

1.1. Swine sector

1.1.1. Pig production and pork consumption

Fast growing animal species with efficient feed conversion rates are needed to support the meat consumption. Modern pig breeds have these characteristics and in fact pork is the most widely consumed form of animal protein, at around 40% of world meat consumption (Figure 1.1) (USDA, 2012) and is the most consumed of all available meats. The three major producers of pork in 2012 year were China with 51.4 millions of tons carcass weight equivalent, European Union with 22.7 millions of tons carcass weight equivalent and United States with 10.5 millions of tons carcass weight equivalent. The major pork exporters are the United States with 2.4 millions of tons carcass weight equivalent, European Union with 2.3 millions of tons carcass weight equivalent, Canada with 1.3 millions of tons carcass weight equivalent, Brazil with 0.6 millions of tons carcass weight equivalent and China with 0.2 millions of tons carcass weight equivalent (USDA, 2012). The major importing countries in 2012 year of the world include Japan, Russia, China, Mexico, Hong Kong, South Korea and United States (USDA, 2012). Commercial pig production has intensified significantly in recent decades. More pigs of the same few breeds are kept on fewer farms, with increased output of animal products (FAO, 2012).

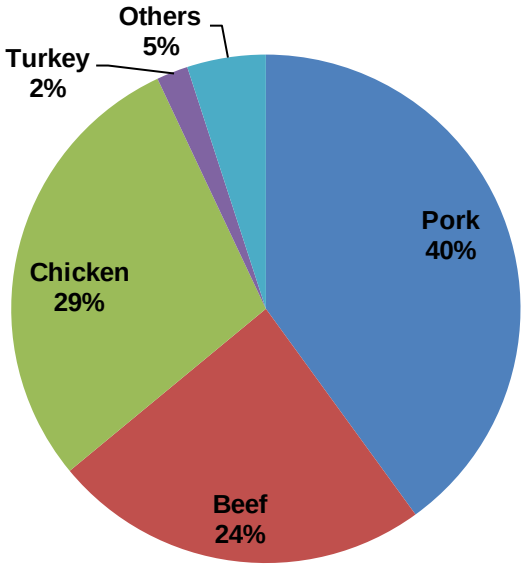

Figure 1.1 – The percentage of the meat consumption in the world. Adapted from USDA Foreign Agricultural Service (2012).

In most European Union countries, pork is main meat produced (Table 1.1). The major producer in EU of pork is Germany, with 5 598 000 and 5 459 000 carcass tons in 2011 and 2012 years, respectively. Only seven countries in EU had higher production of cattle or poultry (*e.g*. Ireland and United Kingdom, respectively) than pork. In Portugal, pork is the main meat produced with 362 342 carcass tons in 2012. However, the degree of self supply is lower in pigs than in poultry, sheep and goats meats in Portugal (Table 1.2).

Table 1.1 – Production of meat in the European Union during 2011 and 2012 years (1000 tons.).

Countries	Pigs		Cattle		Poultry		Sheep and Goats	
	2011	2012	2011	2012	2011	2012	2011	2012
European Union	22388[1]	NA	NA	7537[1]	NA	NA	NA	NA
Belgium	1108	1110	272	262	402	410	2.44	2.16
Bulgaria	48.2	48.8	NA	5.32	98.4	99.1	NA	NA
Czech Republic	263	240	72.1	65.7	170	153	0.16	0.17
Denmark	1718	1603	133	125	186	151	1.50	1.60
Germany	5598	5459	1159	1140	1425	1428	22.0	22.0
Estonia	30.9	NA	7.62	7.24	NA	NA	NA	0.12
Ireland	234	241	547	495	NA	NA	48.1	53.7
Greece	115	115	59.2	56.2	175	182	105	99.4
Spain	3469	3515[1]	604[1]	597[1]	1374	1383[1]	142	133[1]
France	1998[1]	1957[1]	1559[1]	1477[1]	1733[1]	1709[1]	92.7[1]	89.3[1]
Italy	1570	1621	1009	981	1220	1258	33.7	32.2
Cyprus	55.2	51.7	4.82	5.31	27.4	25.3	4.93	5.73
Latvia	23.4[1]	23.9[1]	17.1[1]	16.4[1]	22.8[1]	24.5[1]	0.22[1]	0.34[1]
Lithuania	58.8	58.9	41.1	39.9	75.6	81.7	0.10	0.10
Luxembourg	9.50	10.3	8.88	8.47	0.00	0.00	0.04	0.04
Hungary	387	346	25.9	24.7	383	412	0.16	0.23
Malta	7.26	5.66	1.12	1.11	4.15	4.25	0.07	0.09
Netherlands	1347	1313[1]	381	373[1]	NA	NA	14.7	14.6[1]
Austria	544	530	217	221	NA	NA	8.34	8.37
Poland	1811	1695	380	371	1385	1549	0.61	0.70
Portugal	384	362	96.0	93.0	292	292	10.9	10.6
Romania	263	282	29.1	28.8	294	313	NA	NA
Slovenia	22.9	21.0	35.6	33.1	58.3	58.7	0.12	0.11
Slovakia	56.9	54.1	11.3	9.76	NA	NA	0.53	0.56
Finland	202	193	82.6	80.4	102	107	0.89	0.88
Sweden	256	229	148	135	120	117	5.08	5.04
United Kingdom	806	825	936	883	1557	1608	289	276

This indicator expresses the total carcass weight of pigs slaughtered in slaughterhouses. Source of data: adapted from Eurostat, production of meat (2013). [1]provisional data. NA – not available data on Eurostat.

Table 1.2 – Self supply balance of meats in Portugal in 2007 to 2009 (10^3 tons.).

Years	Intern. Prod.	International live animals market		Prod.	International meat market		Meat available	Meat consum.	Degree of self supply (%)
		Input	Output		Input	Output			
Pigs									
2007	318	75	7	386	157	41	502	492	64.6
2008	332	77	5	404	127	48	483	493	67.3
2009	331	70	5	396	133	44	485	493	67.1
Cattle									
2007	93	2	3	92	108	7	199	197	47.2
2008	109	2	2	109	95	2	202	198	55.1
2009	105	1	3	103	98	4	197	199	52.8
Poultry									
2007	318	-	2	316	34	9	341	338	94.1
2008	326	-	1	325	35	7	353	350	93.1
2009	334	-	1	333	43	10	366	364	91.8
Sheep and Goats									
2007	27	-	1	26	7	1	32	32	84.4
2008	23	-	-	23	7	1	29	29	79.3
2009	19	-	-	19	8	-	27	27	70.4

Source of data: adapted from Instituto Nacional de Estatística (INE), 2009.

1.1.2. Production systems

Pig production and pig meat processing must be conducted in a way that satisfies the expectations and concerns of consumers (Kyriazakis & Whittemore, 2006). In pig production, both intensive (indoor housing) and extensive (outdoor housing) systems are used (Figure 1.2) depending of the objective of production and breed used.

Figure 1.2 – Example of an intensive and an extensive system of fattening. Adapted from www.oje.pt and Nunes & Freitas (2013), respectively.

The objective of intensive system is to produce more meat in less time (normally in 6 months). In the intensive system, the physical environment of a building is specified in terms of temperature, air quality, light and sound. The producers control all the phases of growth to the pigs. Usually, in the intensive systems of production, the weaning is done when the piglets are 28 days of age and with around 7 kg of live weight. Then the phase is the post-weaning until 25 kg of live weight and during around 4 weeks. The pre-growing is the following phase of growing until 60 kg of live weight and during around 7 weeks. Finally, the last phase is the growing-finishing until 90-100 kg of live weight during 8 weeks, and then pigs are slaughtered. Each feed given is adequate needs in each phase of growth pigs in nutrients and energy (Figure 1.3).

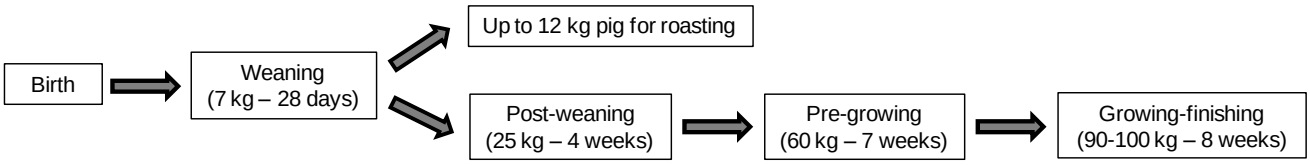

Figure 1.3 – Scheme of intensive system of pig production.

Some breeds are adapted to extensive system, like the Portuguese Alentejano purebred and the Spanish Iberian pigs. This unselected breed have poor productive traits, but a very good adaptation to the environment that allows the extensive production system and the use of natural feeding sources such as the grass and the acorns. In the traditional production system pigs were slaughtered with 140-160 kg of live weight and with 18-24 months of age (Figure 1.4) because for quality characteristics of the meat products an extremely high carcass fat content is required (Lopez-Bote, 1998). There are two periods of calving, the mainly in November/December intended to hams production that the growing-finishing phase is in acorn season. Another calving is in May/June that piglets are for substitution, for sausages industry and meat production. Their meat is used mainly for the elaboration of dry cured meat products of high quality (Nunes, 1993). However, the intensification on agricultural systems and livestock management contributed to a strong decrease on extensive system. In the semi-intensive system, the farms carry out the stages of mating, gestation, lactation, post-weaning and pre-growing. The growing-finishing phase takes place in the typical *montanheira*.

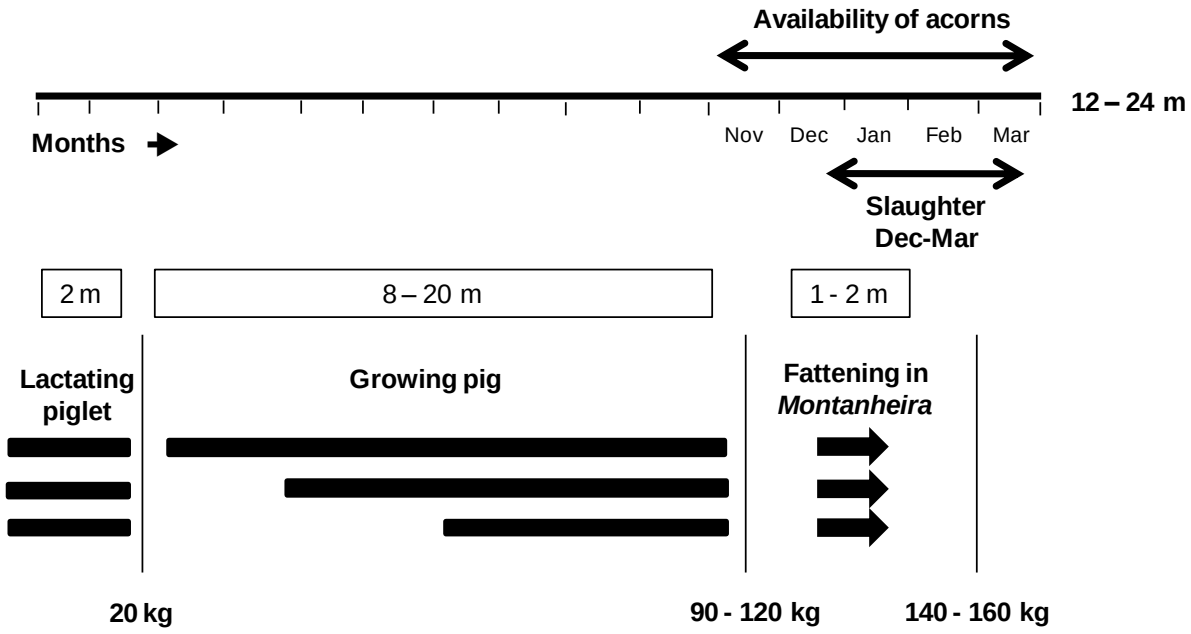

Figure 1.4 – Schematic representation of the production system of Iberian pigs and Alentejano purebred. Adapted from Lopez-Bote (1998).

1.1.3. Pig breeds

Today's pigs are bred and fed to be leaner than the pigs of yesterday. The leaner pork is the result of new technologies in pig production and superior genetics. Producers use the purebred of the major swine commercial breeds, which are: Large White, Landrace, Duroc and Pietrain, to obtain lean meat with high quality. However, in Portugal the autochthonous purebred Alentejano is highly appreciated due to the quality of their meat and their products.

1.1.3.1. Large White breed

The Large White breed of pig was developed in England in the late 1700s. This breed has become well established as a major breed in all pig producing countries. This breed is a rugged and hardy breed that can withstand a wide range of climatic conditions. It is commonly used in crossbreeding or hybrid programs, with the most popular cross being with Landrace. This cross is often used as the maternal line in commercial producers. Breeding programs result in pigs produced for market that meets consumer's requirements of low amounts of fat and high levels of lean meat content. Large White sows have been distinguished for their large litter size, their heavy milk production and for having excellent maternal instincts (Primefacts, 2005). Modern strains are of large size, the females maturing above 300 kg of live weight. Growth rate may readily exceed 750 g daily from birth to 100 kg, producing carcass with 55-60% lean meat. Puberty is at around 180 days, and litter size is

around 11-13 piglets with an average birth weight of about 1.25 kg. The Large White has been subjected to intensive selection against fat over the last 30 years, and is now one of the leanest of all pig breeds (Kyriazakis & Whittemore, 2006).

1.1.3.2. Landrace breed

The Landrace breed was developed in Denmark by crossing the native pig with the Large White. This crossing was then improved during years of testing and breeding under strict government control (Primefacts, 2005). Selected for bacon production and farmed as a single pure breed for many years in Denmark, the Daninsh Landrace is perhaps the most famous of all breeds as an exemplar of the success of progeny testing and selective breeding (Kyriazakis & Whittemore, 2006). This breed improved carcass quality because of the large size of their *longissimus lumborum* muscle. In addition, the Landrace was noted for its early, rapid growth, and its weight at weaning was higher than that of other breeds. Usually it was not as prolific a breeder as the Large White and tended to be slightly fatter (Primefacts, 2005).

1.1.3.3. Pietrain breed

The Peitrain breed originated from Belgium in the 1950's and was later exported to other countries. Pietrains have shorter legs than most breeds, large hams, large loin muscles, high percentage of lean meat in the carcass, light bones and high carcass yield. These pigs are nervous in disposition, they are easily stressed and their meat has a high tendency to PSE (pale, soft and exudative). Their reproductive efficiency is low and they have poorer mothering ability and lower milk production than other breeds. Pietrain produces two fewer piglets per litter when related to other breeds, but can have greater eye muscle area at 100 kg of live weight. These pigs had an effect on the muscle qualities of many European pigs, and their genes have been used to improve the shape of many of the leaner white types (Kyriazakis & Whittemore, 2006).

1.1.3.4. Duroc breed

The Duroc breed is originated from crosses of the Jersey Red of New Jersey and the Duroc of New York. It is known for being red in colour and having floppy ears. This breed is strong in the leg and has an excellent reputation for performing well in tough conditions (Primefat, 2005). It also appears to have a higher proportion of marbling (intramuscular) fat than other genotypes (English, Fowler, Baxter & Smith, 1996). Duroc neither prolific nor lean, it is deeper-muscled but fatter. The use of the Duroc has increased in Europe over recent years,

to improve meat quality when crossed to Landrace, because had higher intramuscular fat in the *longissimus lumborum* muscle. Duroc has slower growth, higher fatness and smaller litter size (Kyriazakis & Whittemore, 2006).

1.1.3.5. Alentejano breed

The Alentejano pig is a Portuguese breed reared in the southern region of Portugal and genetically similar to the Iberian pig. This breed derives from the primitive *Sus scrofa mediterraneum*, which colonised the Iberian Peninsula in the sixth millennium B.C. These pigs have poor productive traits, nevertheless an excellent adaptation to the environment that allows the extensive production system and the use of natural feeding sources such as the grass and the acorns. Until the second half of the 20th century the Alentejano pig was the most important Portuguese swine breed and the main economic product in the majority of the "montados" (oak woods). In the traditional production system pigs were slaughtered with 120-150 kg of live weight and with 18-24 months of age. Their meat was used mainly for the elaboration of dry cured meat products of high quality (Nunes, 1993). Today there is a growing interest towards the use of autochthonous pig breeds reared under sustainable and ecologically friend production system (López-Bote, 1998). Although, this pig breed is characterized by having a slower growth and higher feed conversion ratio and high fat deposition, there is increasingly a growing interest from consumers and producers in their products of higher quality (Ramalho, 2007).

1.1.3.6. Crossbred pigs

The crossbreeding between different breeds of pigs can be a very useful tool for the commercial swine production to increase the efficiency, litter size and improvement of meat quality. The benefits from crossbreeding are successful if choose the combination of available breeds with better productive characteristics. Crossbreeding enables the producer to take advantage of heterosis and to combine desirable characteristics of different breeds. The breeds has different characteristics and a system where males from paternal breeds with superior growth and carcass are mated to females obtain maternal breeds with superior reproduction and mothering ability can take advantage of the strengths of both breeds while minimizing some of the weaknesses. In addition, the crossbred pigs have a higher survival rate than the purebred pigs. The Duroc breed is better in growth rate, intramuscular fat and pork quality, but has more backfat and tends to be used as boars. The Large White and Duroc can both improve productivity and robustness. The Landrace breed offers leanness and increased productivity. The Pietrain breed is commonly used as terminal commercial sires or crossbred pig sires. The cross more commonly used in Portugal is the Large White

gilt mated to the Landrace boar or Large White gilt mated to the Duroc boar. However others crossbreeding are used in commercial production, such as 50% Large White × 50% Landrace gilts (F1) mated to Pietrain or Duroc boars.

1.1.4. Pig nutrition

The objective of the feeding in pig production is to produce piglets from breeding animals, and subsequently meat from those pigs, with a maximum efficiency and profitability. In the pig production, feed is the most costly component and typically represents 60 to 70% of the production costs. For most nutrients, the efficiency with which animals transform dietary inputs to animal products is relatively low (Van Milgen *et al.*, 2012). The correct formulation and rationing of diets for different phases of growth is critical to obtain good results. Energy and nitrogen (amino acids) are the two main components to be balanced in pig diets. Many feedstuffs are appropriate for use in pig diets. Nevertheless, the feedstuffs most common are the barley, wheat, corn and soybean meal.

1.1.4.1. Energy

The majority of the diet cost is associated with meeting the energy needs of pigs. Energy is produced when the carbohydrates, lipid components and protein in feeds are metabolized by oxidative processes in body. Starch is the primary energy source, yielding up its energy after enzymatic digestion in the small intestine, and absorption in the form of glucose. Pigs use the feed energy for the maintenance (muscle movement, digestion, respiration, blood circulation), for milk synthesis, reproductive effort and protein and lipid growth, and for maintaining body temperature (Kyriazakis & Whittemore, 2006). There are different kinds or units of energy used in nutrition such as gross energy, digestible energy, metabolizable energy and net energy. The energy liberated when a substance is combusted in a bomb calorimeter is the gross energy (GE). In feed ingredient, the gross energy concentration is dependent on the proportions of carbohydrate, fat and protein present in the ingredient (NRC, 1998). A typical value for a pig diet based on barley and soya bean would be about 15.5 MJ/kg of diet (English *et al.* 1996). Digestible energy (DE) is the gross energy intake without the gross energy of the excreted faeces, it takes into account the energy components that animal digests. Usually, digestible energy is used to describe the energy requirements of swine and the energy content of swine feeds. Chemical composition of feed ingredients is a major determinant of digestible energy (NRC, 1998). Metabolizable energy (ME) is the digestible energy without gross energy of gaseous and urinary losses (NRC, 1998). Finally, the net energy is the difference between metabolizable energy and heat increment. The heat increment is the amount of heat released because of the energy costs of the digestive and

metabolic processes. Generally, the net energy is the energy that the animal uses for maintenance and production (NRC, 1998). The mainly energy sources (feedstuffs) used in feeds for swine are fat, soybean meal, corn and wheat (Table 1.3).

Table 1.3 – Energy content of some commonly used feedstuffs for swine.

Feedstuff	Dry matter (%)	DE (kcal/kg)	ME (kcal/kg)
Corn	89	3,525	3,420
Barley	89	3,050	2,910
Soybean meal	89	3,490	3,180
Sunflower meal	90	2,010	1,830
Soybean oil	-	8,750	8,400
Wheat	88	3,400	3,250

DE – digestible energy; ME – metabolizable energy. Adapted from Nutrient Requirements of Swine (NRC, 1998).

The growth phases of pigs have different needs of energy. Energy is required for maintenance to sustain basal physiological processes (including thermoregulation and physical activity), growth, pregnancy, lactation and developing boars and gilts. However, energy is also required for the production of animal products. As adenosine triphosphate (ATP), it is involved in the synthesis and degradation of body protein and thus determines protein deposition and lean growth. Excess energy can be stored in the body as lipids (*e.g*., in adipose tissue) (Van Milgen *et al.*, 2012). Feeding the animal in maintenance requirement is if we feed the animal an exact amount of food which will exactly balance its losses of energy (English *et al.* 1996). Only the feed which is provided over and above maintenance can be used for growth. That is established weaned pig can eat about four times as much feed as it requires maintaining itself. At 60 kg of live weight the pigs can eat about three and half times less the maintenance requirements. A characteristic of the need for maintenance energy is that it does not increase as a fixed proportion of body weight but becomes proportionately less in relation to body weight as the animal growth. The feed that is supplied above the maintenance requirement is not converted totally into live weight gain because the process of growth itself requires energy (English *et al.* 1996). For growing pigs, the daily energy requirement is the sum of the energy required for maintenance, for protein and fat synthesis in the lean tissues of the body, and for body heat regulation (Table 1.4). However, genetically superior lean pigs with fast growth rates will require more energy to support the high lean tissue deposition than slower growing pigs. Usually, when pigs are fed a high energy feed (corn-soybean meal) on an *ad libitum* basis, they will consume enough feed to meet their energy requirement.

Table 1.4 – Daily energy requirements and feed intakes of weanling, growing and finishing pigs.

Body weight (kg)	Estimated DE intake (kcal/day)	Estimated ME intake (kcal/day)	Estimated feed intake (g/day)
3 – 5	855	820	250
5 – 10	1,690	1,620	500
10 – 20	3,400	3,265	1,000
20 – 50	6,305	6,050	1,850
50 – 80	8,760	8,410	2,575
80 - 120	10,450	10,030	3,075

DE – digestible energy; ME – metabolizable energy. Adapted from Nutrient Requirements of Swine (NRC, 1998).

1.1.4.2. Protein and amino acids

Pigs from different genetic lines have different protein requirements, which have been associated mainly with differences in lean growth rate. Then, pigs with fast rates of lean growth utilize feed more efficiently and produce carcasses with more muscle and less fat. Therefore, they require a higher concentration of dietary protein (amino acids) to realize their genetic potential for lean growth (Chen, Miller, Lewis, Wolverton & Stroup, 1995).

The amino acid requirements of a growing pig include two components, a requirement for maintenance and a requirement for tissue protein accretion. The principal source of amino acids in pigs is the protein in feedstuffs. Rate of protein deposition is the main determinant of dietary amino acid requirements of growing pigs and is closely associated with feed efficiency and carcass quality. Several factors influence the requirement of dietary amino acids for protein deposition, such as digestion, absorption, post-absorptive metabolism, biological processes (health status, genotype, physiological state) and the environment of the pig (temperature, diet composition, physical environment). Also, genotype, gender, feed intake and live weight influence the amino acid requirements of growing pigs. Through genetic selection pigs at the present deposit relatively more protein and less fat than in the past, mainly because the pressure from consumers to reduce the amount of subcutaneous fat. As a consequence, the requirement for amino acids relative to energy has slowly increased over this time. In pig metabolism, the amino acids have important functions. They are required to build protein in the body, mostly in muscle and to replace protein lost in the course of protein tissue turnover (Kyriazakis & Whittemore, 2006). However, pigs can use dietary amino acids as a source of energy, carbon and hydrogen, to support various body functions or to synthesise body lipid. The feedstuffs for pigs contain approximately twenty amino acids, which nine amino acids are considered essentials. These amino acids (lysine, methionine, threonine, tryptophan, histidine, isoleucine, leucine, phenylalanine and valine) cannot be synthesised by pigs and are required for one or more body functions, and then is

necessary to be supplied in the pig's diet (Kyriazakis & Whittemore, 2006). Nevertheless, the nonessential amino acids are synthesized using carbon skeletons and amino groups derived from other amino acids which are present in excess of requirements (NRC, 1998). However, a few amino acids are not considered essential or nonessential. For example, arginine is generally classified as an essential amino acid, but swine can synthesize arginine, although this synthesis is not adequate to meet nutrient requirements during the early stages of growth (Southern & Baker, 1983). Therefore, the diets of growing swine must contain a source of arginine (NRC, 1998). Nevertheless, swine can synthesize arginine at a rate sufficient to meet most or all of their needs, during postpubertal growth and pregnancy (Easter & Baker, 1976). There are a group of amino acids that are considered the functional amino acids, which include arginine, cysteine, glutamine, leucine, proline and tryptophan (Wu, 2009). These amino acids are important regulators of key metabolic pathways that are necessary for maintenance, growth, reproduction and immunity in organisms, thus maximizing efficiency of feed utilization, enhancing protein accretion, reducing adiposity and improving health (Suenaga, Tomonaga, & Yamane, 2008). In pig diets, the first limiting amino acid is lysine, and then is necessary to supply this amino acid in all diets for pigs. However, there are other amino acids that are necessary included in pig diets may become limiting as well, particularly threonine, methionine, cysteine, tryptophan and isoleucine. The amino acids were destined for pig protein, but they are also useful precursors for other body activities, for example the formation of fatty tissues (Kyriazakis & Whittemore, 2006). In pigs, there are few characteristics clinical signs of amino acid deficiencies, which are a reduction in feed intake that is accompanied by increased feed wastage. However, pigs can tolerate high intakes of protein, but the excess of 25 percent protein to growing-finishing pigs is wasteful, contributes to environmental pollution and results in a reduced weight gain and feed efficiency (NRC, 1998). On the contrary, addition of excessive supplements of amino acids, such as arginine, leucine and methionine can reduce feed intake and growth rate (Brudevoid & Southern, 1994). For growing-finishing pigs, amino acids requirement are influenced by their genetic capacity to deposit body protein (NRC, 1998).

Protein metabolism requires a supply of "ideal protein", that is essential amino acids balanced correctly for the various purposes of maintenance and production. The value of a diet protein depends upon the relationship between the balance of essential amino acids required to constitute ideal protein and balance of amino acids supplied from the diet (Kyriazakis & Whittemore, 2006). Ideal protein is conceived as providing the essential amino acids in the proportions required by the pig and of having the correct balance between the essential and non-essential amino acids. The balance of amino acids in the ideal protein related to maintenance, growth of lean tissue, pregnancy and lactation will be different,

reflecting differences in the composition of ideal protein (McDonald, Edwards, Greenhalgh & Morgan, 2002). Proteins ingested by pigs are subject of two processes, the enzymatic digestion in the stomach and small intestine and microbial fermentation, which occurs largely in the hind gut. Therefore, enzymatic digestion involves the breakdown of protein to small peptides and free amino acids which can be absorbed by intestinal cells, although absorbed peptides are hydrolysed to free amino acids within intestinal cells. These cells will utilise some amino acids, but most absorbed amino acids are released into the blood circulation. However, the ileal digestibility, that is the digestibility prior to the end of the small intestine, which provides a better estimate of amino acid bioavailability in feedstuffs for pigs than faecal digestibility (Kyriazakis & Whittemore, 2006).

Cereal grains, such as corn, barley or wheat are the primary ingredients used of the most pig diets and usually provide 30 to 60 percent of the total amino acid requirements (NRC, 1998), but the concentration of protein is low, about 8-12% (Kyriazakis & Whittemore, 2006). Consequently, the diet requires to be supplemented with higher protein seeds, for example with soya bean and oilseed rapeseed with 20-30% protein. There is a large variability in protein and amino acid contents across feedstuffs (Table 1.5).

Table 1.5 – Protein and amino acid composition of the most commonly used feedstuffs for swine.

Feedstuff	Crude protein (%)	Lys (%)	Met (%)	Thr (%)	Trp (%)	Ile (%)
Corn	8.3	0.26	0.17	0.29	0.06	0.28
Barley	11.3	0.41	0.20	0.35	0.11	0.39
Soybean meal	43.8	2.83	0.61	1.73	0.61	1.99
Sunflower meal	26.8	1.01	0.59	1.04	0.38	1.29
Wheat	14.1	0.38	0.23	0.41	0.16	0.47

Adapted from Nutrient Requirements of Swine (NRC, 1998).

1.1.4.2.1. Lysine

Lysine is an essential amino acid, and the first limiting amino acid in pig diets and it is common to include synthetic lysine in the diet of pigs to meet their requirement (Wu, 2009). The requirement for each of the essential amino acids follows a concept referred to as ideal protein. The requirement of the growing pig for dietary lysine as well as its response to change in energy intake is determined largely by its capacity for muscle growth (English *et al.* 1996). In practice, pig diets are formulated to ileal-digestible lysine, and the ileal-digestible contents of the other essential amino acids in the diet are maintained in the same proportion to lysine as they are in the ideal protein (McDonald *et al.*, 2002). In view of the fact that ileal digestion of ingested proteins is both incomplete and variable, ileal digestibility is a means to

assess routinely amino acid availability in feedstuffs for pigs. However, ileal amino acid digestibility is influenced by animal factors as well as feed factors (Kyriazakis & Whittemore, 2006). To define the true digestibility of the dietary amino acids, the two streams of undigested amino acids, dietary and endogenous, passing the end of the ileum must be separated. True digestibility is always higher than apparent digestibility (Kyriazakis & Whittemore, 2006). However, is necessary to determine the lysine requirements for each growth phases of pigs, because this amino acid is the first-limiting amino acid in diets for pigs. In the growing-finishing pigs, the daily lysine requirement is the sum of the requirements for maintenance and for protein accretion (NRC, 1998). The daily true ileal digestible lysine requirement for maintenance is assumed to be 0.036 g per metabolic body weight ($BW^{0.75}$) (Wang & Fuller, 1989). The requirement for lysine may be translated into that for the replacement of lost protein using the presumption of 65 g lysine per kg protein (Fuller, McWilliam, Wang & Giles, 1989), giving a lower estimate of 0.55 g protein per $W^{0.75}$. There are two components that support the protein accretion for daily amount of lysine, which are the daily amount of protein accreted and the amount of true digestible lysine needed for each gram of protein accreted (NRC, 1998). However, the protein accretion rate can be calculated to different ways. This mean accretion rate can be calculated from carcass weight at slaughter, percent fat-free lean in the carcass at slaughter, assumed fat-free lean in the carcass at the beginning of the growing period and the number of days in the growth period. For each estimate, the amount (g/day) for true ileal digestible lysine above maintenance consumed at the requirement was divides by the protein accretion rate (g/day) at that level of lysine intake. Therefore, the relationship of lysine required above maintenance to whole-body protein accretion rate is as follows: Lysine = 0.12 x PD, where lysine is the daily requirement for true ileal digestible lysine intake above maintenance in grams, and PD is daily protein deposition in the whole-body in grams (NRC, 1998). This calculation of lysine estimation is valid to commercial pig breeds and the requirements for lysine calculated for different phases of growth in NRC (1998) are presented in Table 1.6. For other breeds, such as Alentejano purebred pig, the lysine requirements can be calculated with protein deposition value 0.71 g/day (Barea, Nieto & Aguilera, 2007; García-Valverde, Barea, Lara, Nieto & Aguilera, 2008). However, the efficiency of lysine utilization depends on the supply of other essential amino acids and the sum of non essential amino acids, when their ratios to lysine fall below critical values (Susenbeth, 1995).

Table 1.6 – Dietary lysine requirements of growing pigs.

Body weight (kg)	Lysine (True ileal digestible basis %)
3 – 5	1.34
5 – 10	1.19
10 – 20	1.01
20 – 50	0.83
50 – 80	0.66
80 – 120	0.52

Adapted from Nutrient Requirements of Swine (NRC, 1998).

1.1.4.2.2. Arginine

Arginine is considered a functional amino acid, and is essential in the early stages of growth of mammals. Arginine has a large number of functions in nutrition and metabolism, directly and nitric oxide via. Directly, arginine has the function of activation mammalian target of rapamycin (mTOR) signaling, antioxidant, regulation of hormone secretion, ammonia detoxification, regulation of gene expression, immune function and methylation of proteins. However, nitric oxide via arginine has the function of signaling molecule, regulator of nutrient metabolism, vascular tone, hemodynamics, angiogenesis, spermatogenesis, embryogenesis, fertility, immune function, hormone secretion, wound healing, neurotransmission, tumor growth and mitochondrial biogenesis (Wu, 2009). Arginine is the physiological precursor for the synthesis of NO, which stimulates the oxidation of fatty acids and glucose in a cell-specific manner (Jobgen, Fried, Fu, Meininger & Wu, 2006). Also, arginine might affect multiple metabolic pathways involving fatty acid and glucose synthesis, amino acid degradation, and cellular redox state (Wu *et al.*, 2009; Wu, 2009). In mammals, growing evidence shows that arginine plays an important role of regulating the metabolism of energy substrates and, consequently, nutrient partitioning (Jobgen *et al.*, 2006). However, physiological levels of nitric oxide stimulate glucose uptake and oxidation, as well as fatty acid oxidation in insulin-sensitive tissues (muscle, heart, liver and adipose tissue), inhibit the synthesis of glucose, glycogen and fat in some tissues, such as liver and adipose tissue, and enhance lipolysis in adipocytes (Figure 1.5) (Jobgen *et al.*, 2006).

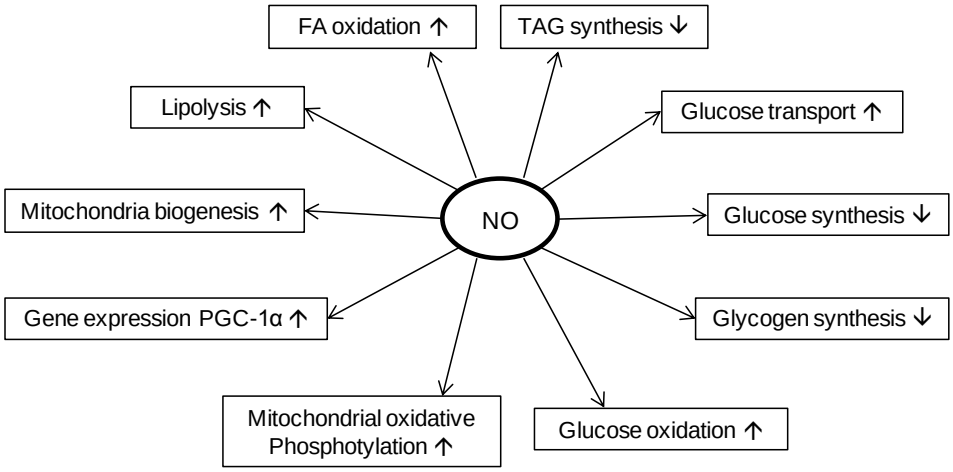

Figure 1.5 – Overall effects of physiological levels of nitric oxide (NO) on energy substrate metabolism. Adapted from Jogben *et al*. (2006). ↑, increase; ↓, decrease; TAG, triacylglicerols; PGC-1α, Peroxisome profliferator-activated receptor gamma coactivator 1α.

Glutamine, glutamate, proline, aspartate, asparagine, ornithine, citrulline and arginine are called the arginine family of amino acids that are interconvertible via complex interorgan metabolism in most mammals, including pigs (Figure 1.6) (Wu *et al.*, 2007). Although arginine is formed in the liver via the urea cycle, there is no net synthesis of arginine by this organ (Urschel, Shoveller, Pencharz & Ball, 2005) due to an axceedingly high activity of cytosolic arginase that rapidly hydrolyzes arginine (Wu & Morris, 1998).

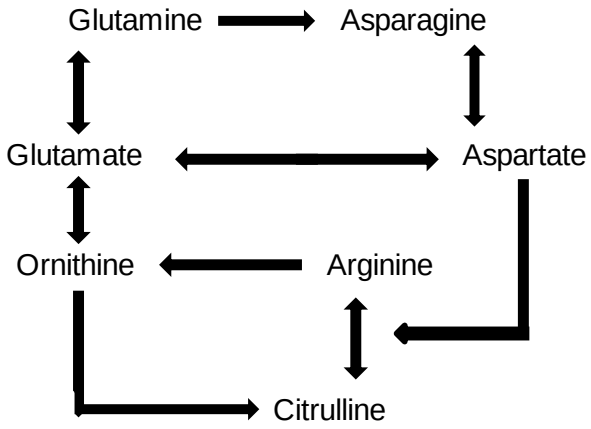

Figure 1.6 – Interconversion of the arginine family of amino acids in neonatal and post weaning pigs. Adapted from Wu *et al.* (2007).

1.1.4.2.3. Leucine

Leucine is an essential amino acid and has the function of regulation of protein turnover through cellular mammalian target of rapamycin (mTOR) signaling and gene expression, activator of glutamate dehydrogenase, branched-chain amino acids (BCAA) balance and flavour enhance (Wu, 2009). For protein synthesis, leucine serves a substrate, and increases tissue protein synthesis of weanling piglets fed a low protein diet (Yin *et al.*, 2010). Also, leucine affects satiety by stimulating of leptin secretion and regulates the protein synthesis in skeletal muscle and in the other tissues including the adipose tissue (Lynch *et al.*, 2006). The regulation of mTOR signaling depending on both ribosomal protein S6 kinase (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1) phosphorylation. It also stimulates mTOR resulting in decreased food intake, weight gain and fat mass. For the moment, leucine affects satiety by stimulating of leptin mRNA translation and secretion. The activation of mTOR pathway by leucine has been shown to activate insulin receptor substrate 1 (IRS-1) and impair phosphatidylinositol 3-kinase (PI3K) activity (Figure 1.7) (Li, Yin, Tan & Kong, 2011).

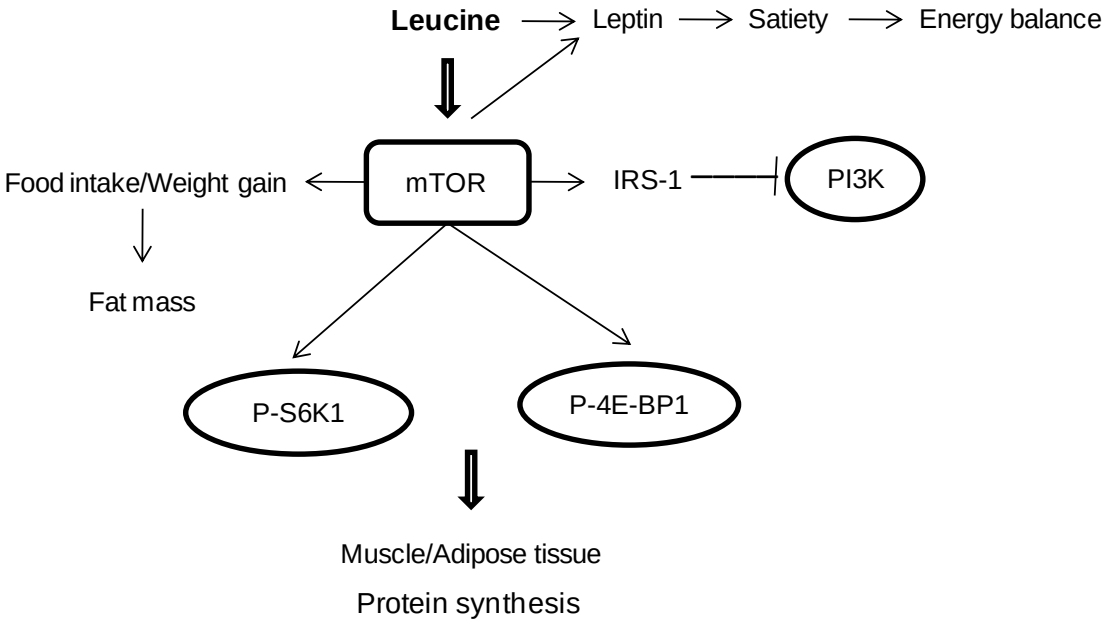

Figure 1.7 - Role of leucine in the regulation of mTOR signal pathway. Adapted from Li *et al.* (2011). IRS-1, insulin receptor substrate 1; PI3K, impair phosphatidylinositol 3-kinase; P-S6K1, ribosomal protein S6 kinase; P-4E-BP1, eukaryotic initiation factor 4E binding protein 1.

When leucine is added to dietary animals or humans, this amino acid significantly improves glucose metabolism. Leucine provides the substrates for gluconeogenesis and regulates oxidative use of glucose by skeletal muscle during stimulating of glucose recycling via the glucose alanine-cycle. This amino acid also plays critical roles in muscle protein loss and enhancing glycemic control, and regulates adipocytes lipid metabolism to promote partition of lipid to skeletal muscle (Figure 1.8) (Li *et al.*, 2011), and mitochondrial biogenesis is involved in mediating this effects. Also, leucine plays critical roles in adipocyte and skeletal muscle energy metabolism (Sun & Zemel, 2007).

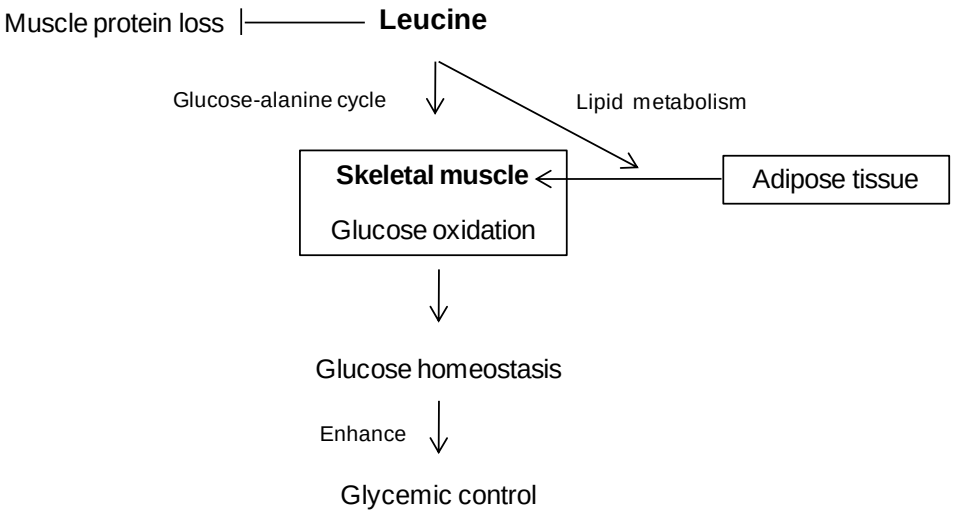

Figure 1.8 – Potential role of leucine in the regulation of glucose homeostasis. Adapted from Li *et al*. (2011).

1.2. Pig carcass and pork quality

Pork quality has become a primary focus for producers, researchers, processers, retailers, and ultimately, consumers. Animal factors, such as breed, muscle type, fat, ultimate pH, colour, and environmental factors, including pre-slaughter conditions, housing, exercise and post-slaughter processes, such as, electrical stimulation, chilling and cooking, affect the pork quality.

1.2.1. Pig carcass traits

Pig carcass quality depends on some factors, such as breed selection and nutrition. Over the last 25 years, the most dramatic aspect of improvement in pig meat quality is fat reduction. Pig carcasses have become leaner over an improved understanding of nutritional

requirement and over breeding selection for lean types (Kyriazakis & Whittemore, 2006) for the consumers demand. The major criterion of assessment of quality in pig carcasses is the backfat depth, usually at the P2 site in the last rib position, which is the most representative location (Teye *et al.*, 2006). Generally, the best eating quality is obtained at levels of fatness associated with P2 backfat depths at 100 kg live weight of between 8 and 14 mm. At below 8 mm the quality of the lean falls, while above 14 mm the meat is excessively fatty (Kyriazakis & Whittemore, 2006). The chemical composition of the entire male meat pig with the empty body weight of 100 kg and measurement at the P2 backfat dept of 10 mm will be around 67% water, 17% protein, 13% lipid and 3% ash. Usually, the carcass weight of a pig is between 70 and 80% of its live weight. Carcass yield is the proportion of the weight of the commercial carcass (without giblets) recovered from the body live weight and is expressed in percentage. The major influences upon carcass yield are the live weight, fatness and genotype. At the weights of 100-120 kg values are 80% approximately (Kyriazakis & Whittemore, 2006).

1.2.2. Meat physical traits

1.2.2.1. Pork pH

One of the most important factors for eating quality of meat is the *post-mortem* ultimate pH (pHu). Some studies indicated that a higher pHu results in an increased tenderness (Eikelenboom & Hoving-Bolink, 1994b). The results obtained for Huff-Lonergan *et al.* (2002) about pork quality traits indicate that a lower pHu was associated with lighter-colored, with higher drip loss, less tender and with less pork flavour and more off-flavour in the meat. Other factor that could be a result in lower pHu is higher amounts of glycogen in the tissue at slaughter that can provide the potential for a sustained glycolysis in the muscle after slaughter (Monin & Sellier, 1985). Lonergan *et al.* (2007) confirms in their results that pHu has a significant role in determining the pork sensory quality. The pHu is often associated to pale, soft and exsudative (PSE) and dark, firm and dry (DFD) meats. After slaughter, while the carcass is warm, energy metabolism lowers the muscle pH, because muscle glycogen goes to lactic acid. In general, the pH falls gradually, reaching about 5.5, 24 hours after slaughter. Nevertheless, when the pHu is to low and the muscle metabolic activity is prolonged or excessively rapid with a simultaneous high muscle temperature, there are the rapidity of the acidification of the muscle that cause PSE meat. PSE is a result of soluble proteins precipitating, the muscle fibre cell membranes leaking as they become depleted of energy, and intramuscular fluid rich in lactic acid being exuded from within the fibres. This process occurs when muscle shows a pH of less than 5.8 measured 45 minutes post-

slaughtered; when the normal values pH_{45} are 5.8-6.4 (Kyriazakis & Whittemore, 2006). Moreover, the combination of low pH and high temperature accelerates the inactivation of the oxygen-consuming mitochondrial enzymes and promotes the oxygenation of the muscle pigment to the bright red oxymyoglobin (Govindarajan, 1973). However, the PSE can also occur due mishandling and pig stress. Dark, firm and dry muscle is less commonly happened and is attributed to poor handling between the farm and abattoir. When occurs the DFD meat pH tends not to fall and to remain around or above 6.5, 45 minutes post-slaughter, and to have a pHu of above 6.0 (Kyriazakis & Whittemore, 2006). DFD meat is a result of limited muscle glycogen stores at the time of slaughter, which results in high final pH of the meat (>6.0) (Lindahl, Henckel, Karlson & Andersen, 2006). At these high pH values, endogenous oxygen-consuming enzymes in the meat are active, which promotes the formation of the purple, reduced muscle pigment deoxymyoglobin (Ledward, 1992).

1.2.2.2. Pork colour

Colour is an important quality consideration attributed to fresh pork. Colour perception plays a major role in consumer evaluation of meat quality (Lanari, Schaefer & Scheller, 1995). The colour of pork is, by the consumer, strongly associated with the expected meat quality (Bredahl, Grunert & Fertin, 1998) and the consumer prefers pork with a high intensity of pink (Brewer, Lan & Mckeith, 1998). The meat colour is influenced by different factors, such as breed, gender, production, handling and slaughter conditions (pH and temperature). The pigment content, the chemical form of the pigment and the meat structure affect the colour of pork (Warris, Brown, Adams & Lowe, 1990a). Meat colour is dependent on myoglobin content. Myoglobin is a water-soluble protein that stores oxygen for aerobic metabolism in the muscle. A higher myoglobin concentration yields a more intense colour and this concentration differs between animal species. For example, pork has lower myoglobin than beef, thus giving it a less intense colour. Also, different muscles of pork have different proportions of myoglobin. Red oxidative muscles (*rectus femoris* muscle and *biceps femoris* muscle) have higher myoglobin content than white glycolytic muscles (*longissimus dorsi* muscle and *gluteus medius* muscle). There are three myoglobin forms that are dependent on the oxygen contact and affect the colour hue of fresh pork. The oxymyoglobin, when myoglobin is oxygenated, associated with bright pink (red) colour, the metamyoglobin, when myoglobin is oxidized, associated with brown colour, and finally desoxymioglobin, when myoglobin is reduced and is associated with purple colour (Lindahl, Lundstrom & Tornberg, 2001). Also, high pHu can affect the colour stability of fresh meat because it affects enzyme activity and the rate of oxygenation. The high pHu as a dry surface and this inhibits the oxygen contact into the meat and thus slows down the oxygenation process. During the

conversion of muscle to meat, extreme progress in pH has long been known to influence the colour characteristics of pork. This fact provided the basis for two inferior meat quality grades, DFD and PSE meat (Lindahl *et al.*, 2006). The combination of low pH and high temperature accelerates the inactivation of the oxygen-consuming mitochondrial enzymes and promotes the oxygenation of the muscle pigment to the bright red oxymioglobin (Govindarajan, 1973). Colour can be measured by the CIELAB recommendations (C.I.E., 1978) and by the three principle colour dimensions: L* (lightness), a* (redness) and b* (yellowness). Bidner *et al*. (2004) found that about 70% of the variation in L* and b* values, but only 28% of the a* value in the *longissimus dorsi* muscle was explained by pHu using a range (4.86-7.15). Another study reported that about 40% of the variation in the L*, a* and b* values was explained by temperature and pH measurement during the first 2 hours *post-mortem* (Lindahl *et al.*, 2006). Lindahl *et al.* (2001), reported that higher a* values, as well as the higher L* and b* values, could be explained by a less reductive environment that promotes oxygenation of myoglobin to oxymyoglobin. On the other hand, lower a* and b* values are related to more deoxymyoglobin at the meat surface, in the other words, to a lower degree of myoglobin oxygenation. The gender of pigs can be influenced the meat colour. Warris *et al.* (1990a) found higher a* values and more redness of meat in gilts when compared to castrates male pigs. Additionally, Jeremiah *et al.* (1999) found a slightly darker colour in *longissimus lumborum* muscle from gilts, when compared to castrates male pigs. Pork colour is also affected by genetics factors. Brewer *et al.* (2004) evaluated several sire lines and reported that genetic line affected the lightness, pinkness and a* value. In another study, the results suggested that Duroc breed had more favourable visual colour, higher pH and increased redness than Pietrain sired pigs (Edwards, Bates & Osburn, 2003). The diet or different supplementation in diets can influence the meat colour. Some studies have shown that dietary supplementation with certain nutrients may improve water-holding capacity, colour and anti-oxidative capacity of pork muscle. These nutrients have antioxidant function and include magnesium, selenium, vitamin E, vitamin C, tryptophan, creatine and conjugated linoleic acids (Bukley, Morrissey & Gray, 1995; Swigert, Mckeith & Carr, 2004).

1.2.2.3. Pork sensory attributes

The most critical characteristics for eating quality of pork are flavour, tenderness and juiciness, which influence the choice of consumers. The sensory attributes of pork could be affected by many factors, such as breed, gender, carcass weight, diet, genetic variation and biochemical changes that occur during slaughtering, cooling routines, maturation and cooking methods (Flores, Armero, Aristoy & Toldra, 1999). Sensory attributes are defined as appearance, odour/aroma/fragrance, flavour, texture and specific feeling/chemical factors.

Texture can be defined as the sensory and functional manifestation of the structural and mechanical properties of meat, detected through the senses of vision, hearing, touch and kinaesthetic (Szczesniak, 1963) and consisted of hardness, cohesiveness, adhesiveness, denseness, springiness, perception of particles and perception of water.

Tenderness is one of the most important textural properties and the most important sensory attributes when discussing the acceptance of meat and is one of the major quality attributes of meat. Tenderness of meat may be simply defined as the facility which a piece of meat can be cut and chewed (McKee, 2007). Meat tenderness is influenced by main factors such are connective tissue, intramuscular fat content and myofibrillar structure (Van Laack, Stevens & Stalder, 2001). Tenderness variation arises mainly through changes to the myofibrillar protein structure of muscle in the period between animal slaughter and meat consumption (Wood *et al.*, 1999). Several studies show that intramuscular fat was positively correlated with tenderness of meat. Fernandez, Monin, Talmant, Mourot & Lebret (1999a) reported that increasing intramuscular fat from 1.25 to 3.25% resulted in a trend to increasing tenderness. DeVol *et al.* (1988) found that tenderness was significantly correlated with intramuscular fat when evaluated by trained taste panel. The ultimate pH could be determinant on tenderness. Eikelenboom & Hoving-Bolink (1994b) indicated, that in pork, a higher pHu results in increased tenderness. The muscle type differs on lipid concentration and is generally higher in red muscles than in white muscles and the fibres types are different. The *psoas major* muscle in comparison to the *longissimus lumborum* muscle is significantly tender, which suggests that marbling fat is a marker for muscle fibre type and associated metabolic differences (Wood *et al.*, 1999). The external factors, such as breed and diet also affect the sensory characteristics, namely the tenderness. Warris, Kestin, Brown & Nute (1990b), in a comparison with several pig breeds showed that the traditional breeds, such a Duroc and Large White breeds, tended to have higher muscle lipid concentrations and more tender meat than modern lean breeds. Blanchard, Chadwick, Warkup, Ellis & Deans (1995) reported that pigs fed diets with high-energy and low-protein, produced meat more tender. Fast growth and their implication in higher protein turnover (and proteolysis) can be an explanation to improve meat tenderness (Wood *et al.*, 1999).

Juiciness is a sensory term, which refers to mouth feel of the moisture released from meat during mastication. Thus, juiciness is indicative of the moisture content in meat, which is critically affected by the water-holding capacity as well as the hydration ability of meat (McKee, 2007). The juiciness facilitates de chewing process as well as brings the flavour component in contact with the taste buds (Aaslyng, Bejerholm, Ertbjerg, Bertram & Andersen, 2003). The juiciness of meat depends on the raw meat quality and on the cooking procedure. Some studies have been reported that juiciness is positively correlated with

intramuscular fat, but more correlated with ultimate pH (Eikelenboom, Hoving-Bolink, & Van der Wal, 1996). The water holding capacity of meat might also influence the juiciness (Hamm, 1972). The high glycogen concentration could also increase the juiciness. The juiciness can be influenced by the rearing conditions. The meat from indoor reared pigs has been shown higher juiciness than meat from pigs reared outdoor (Jonsall, Johansson & Lundstrøm, 2001).

Flavour includes aromatics, tastes and chemical feelings. Aromatics consist of smell perceptions caused by volatile substances released from meat in the mouth. Tastes consist of salty, sweet, sour and bitter perceptions caused by soluble substances in the mouth and chemical feelings include astringency, spice heat, cooling and metallic flavour (McKee, 2007). Flavour, as an attribute of meat, has been defined as the perceptions resulting from stimulation of the sense ends that are grouped together at the entrance of the alimentary and respiratory tracts (Meilgaard, Civille & Carr, 1991). It is a complex group of sensations comprising of olfactory, taste and other chemical heat (Lawless & Heymann 1998). Meat flavour is influenced by genetics (species) and environment (feed) factors. The meaty flavours of cooked meat are produced in reactions between carbohydrates and proteins, and between breakdown products of these compounds (Mottram, 1992). Lipids also contribute to flavour through their degradation products, such as, aldehydes, alcohols and ketones. Age also increase flavour intensity in meat animals, is to be due to changes in tissue constituents. In pigs, restricting dietary intake during the growth period or few weeks earlier to slaughter had a determinant effect on pork flavour, while protein level in feed had no effect (Melton, 1990). Feeding fish meal, fatty acids or others supplements can be resulted in pork products with undesirable flavour.

Off-flavour is the term used to describe unpleasant odours or tastes resulting from the natural deterioration of a meat, with extensive deterioration giving rancidity. Diet manipulation could contribute to off-flavours appearance that can be with lipid oxidative stability and rancidity. In the entire male pigs, the “boar taint” produces abnormal odours and flavours in meat, which is can be considered the off-flavour.

The meat quality traits, which affect the sensory attributes, the ultimate pH is the most highly correlated with tenderness, juiciness, flavour and off-flavour, that are expected to impact the extent of pH decline, such as residual glycogen, lactate and glycolytic potential (Huff-Lonergan *et al.*, 2002). The overall acceptability is the classification that panellist give as a sum of the perceptions of tenderness, juiciness and flavour of meat.

1.2.3. Pork lipids

1.2.3.1. Intramuscular fat

Intramuscular fat (IMF) is the quantity of fat within muscles, while intermuscular fat is the fat located between different muscles in a same cut. IMF in pork usually is not visible and it is not anatomically separable. Chemically, IMF is the sum of phospholipids (principally found in cell membranes), triacilglycerols (principally forms of energy reserves) and cholesterol (Hocquette *et al.*, 2010). IMF content varies between species, breeds and muscle types in the same animal. The lipid content in muscles of the loin is about 1.5 to 2%. Among the most common breeds of pigs, the Duroc is the breed that has higher values of lipid content, about 2.4% (Mourot & Hermier, 2001).

For farm animals, such as pig, the major breeding goals have been to reduce carcass fatness. The reason for that is the consumer's preference for meat with a minimal amount of visible fat. Some factors, such as gender, age and feeding of animals are involved in the variation of IMF. In slaughter pigs, the boar is leaness than gilts or barrows (De Smet, Raes, Demeyer, 2004). Lebret, Juin, Noblet & Bonneau (2001) reported that IMF content was reduced in older pigs, but that had no influenced eating quality of pork. The IMF content, also vary with the number and size of intramuscular adipocytes. The amount of IMF and its fatty acid composition play an important role in the quality of pork, including sensory attributes. Several reports concluded that IMF content positively influences sensory quality traits, as tenderness, juiciness and flavour of meat and overall acceptability, while a low amount of IMF is associated with a less tasty meat (Hodgson, Davis, Smith, Savell & Cross, 1991; Wood *et al.*, 2008). Eikelenboom & Hoving-Bolink (1994a) found that higher IMF was associated with better pork eating quality. DeVol *et al.* (1988) suggested that acceptable pork eating quality requires a minimum IMF of 2.5 to 3.0%. More recently, Fernandez, Monin, Talmant, Mourot & Lebret (1999b) reported that flavour and juiciness were significantly improved when IMF levels increased above approximately 2.5%.

Some feeding strategies have been used to increase IMF and consequently, to improve meat quality. Conjugated linoleic acid (CLA) supplementation (Dugan, Aalhus, Jeremiah, Kramer & Schaefer, 1999; Gatlin, See, Larick, Lin & Odle, 2002), the use of RPDs (Doran *et al.*, 2006), low lysine levels (D'Souza *et al.*, 2008), leucine (Cisneros *et al*., 1996; Hyun *et al.*, 2003) and arginine (Ma *et al.*, 2010) supplementation have been shown to improve meat quality increasing IMF content levels in pigs.

1.2.3.2. Fatty acid composition of pork

Muscle contains mostly water and protein with little lipid, which is located primarily in muscle cell membranes as well as in small lipid droplets associated with red or oxidative fibers (Rule, Smith & Romans, 1995). Fatty acids are compounds synthesised in nature *via* condensation of malonyl coenzyme A units by a fatty acid synthase complex. They usually contain even numbers of carbon atoms in straight chains (commonly C_{14} to C_{24}), and may be saturated or unsaturated, and can contain a variety of substituent groups (Christie, 2010).

Fatty acids can be classified as saturated fatty acids (SFA), which contain no double bounds, MUFA, which feature one double bound, and polyunsaturated fatty acids (PUFA), which contain multiple double bonds. Fatty acid composition of IMF has a considerable impact on eating quality and human health. Fatty acid composition and total amount of SFA have been identified as dietary risk factors, related to various cancer and especially coronary heart disease (Pascual *et al.*, 2007). One research in 14 European countries documents average SFA levels of 34-52% fatty acid methyl ester (FAME) for pork. In pork, the more abundant saturated fatty acids are myristic acid (14:0), palmitic acid (16:0) and stearic acid (18:0) (Schmid, 2011).

The MUFA in pork fat are generally around 40-50% of the fat. Overall, the most frequently monounsaturated fatty acid found in pork fat is the oleic acid (18:1*n-9*). In pork, the PUFA levels are approximately 10-20%. The major PUFA include linoleic acid (18:2*n-6*), linolenic acid (18:3*n-3*) and arachidonic acid (20:4*n-6*). In pork, the proportion of PUFA in intramuscular fat is generally higher than in beef and easier to manipulate by dietary factors (Wood & Enser, 1997).

The human dietary recommended P/S ratio (ratio of PUFA to SFA) is 1-1.5 that is considered beneficial, but in meat this ratio is generally unfavourable, as the SFA far outweigh the PUFA (Schmid, 2011). Wood *et al*. (2003) recommended P/S ratio should be increased to above 0.4. Nevertheless, in pork this ratio can be changed when including some source of polyunsaturated fat in the pig's diet. Although animal products with a higher linoleic acid or *n*-3 PUFA content may be desirable, the impact of these fatty acids on meat quality is a concern due to the higher potential for oxidative damage, off-flavors and in pork the effect on carcass firmness (Hausman *et al*., 2009).

In monogastrics, the PUFA content can be influenced by dietary factors, it is diluted by the novo fatty acids synthesis consisting of SFA and MUFA, thus causing a decline in the P/S ratio with an increasing fat deposition (De Smet *et al.*, 2004). Another important aspect of meat PUFA composition is the ratio *n-6* to *n-3* fatty acids, which is generally much higher in pork than in beef or veal. The recommendation for this ratio is less than 4, but some meats have higher values (Wood *et al.*, 2003). This value is difficult to reduce due to the high

content of C18:2 in the cereal-based and this produce an undesirably high *n-6*/*n-3* ratio (Wood *et al.*, 2003).

Several factors can modify the fatty acid composition of fat deposits, such as breed, muscles, gender and diet. The fatty acid composition can be different between breeds. For example, the Duroc pig breed has higher muscle lipid content comparatively with other breeds (Wood *et al*., 2004). Cameron & Enser (1991) reported that IMF of Duroc contained more saturated (14:0 and 16:0), and less PUFA (18:2, 20:4, 22:5 and 22:6) than Landrace pigs. Other study with Large White and Pietrain breeds showed that total lipids of Large White contained more SFA, particularly 18:0 and less PUFA, particularly 18:2 and 18:3 than Pietrain (Monin, Hortós, Diaz, Rock & Garcia-Regueiro, 2003).

Fatty acid composition is different between muscles due differences in muscle fibre type. The "red" muscles such as *psoas major* have a higher proportion of phospholipids than "white" muscles such as *longissimus lumborum*, and therefore a higher percentage of PUFA (Wood *et al.*, 2003). Some studies have reported that gender can be affecting the fatty acid composition of meat. Alonso, Campo, Español, Roncalés & Beltrán (2009) reported that gender had a little influence in fatty acid composition of IMF. SFA and MUFA no altered between genders, but have a slight tendency for PUFA to be higher in gilts than in castrated males.

Pigs are monogastric animals and many dietary components are consequently transferred from the feed to the muscle and fat tissues and consequently affect the fatty acid composition and pork quality (Rosenvold & Andersen, 2003). Fatty acid composition also varies according to the rearing system of pigs (Gandemer, 2002). Various authors reported that meat of pigs reared in "montado" are characterised by a high level of unsaturated fatty acids (Andrés *et al.*, 2001; Cava, Ventanas, Tejeda, Ruiz & Antequera, 2000). In pork, fatty acid composition can be changed with some strategies of feeding such as oils supplementation (Teye *et al.*, 2006) and RPDs (Doran *et al.*, 2006).

The fatty acid composition has an important effect on meat quality, such as fat tissue firmness, shelf life (oxidation) and flavour. The effect of fatty acids on firmness of meat is due to the different melting points. The shelf life is influenced by fatty acids because the propensity of unsaturated fatty acids to oxidise, leading to the development of rancidity as display times increases. The effect of fatty acid on meat flavour is due to the production of volatile compounds, odorous, lipid oxidation and products during cooking (Wood *et al.*, 2003).

1.2.3.3. Lipid content and fatty acid composition of adipose tissue

Adipose tissue is composed mainly of fat because its main function is to store lipid. However, adipose tissue is also an endocrine organ in which adipocytes secrete several factors that affect other tissues (Kokta, Dodson, Gertler & Hill, 2004). In addition to stored lipids, adipose cells also are delimited by membranes that contain structural lipids (Rule *et al.*, 1995). The adipose tissue is constituted of a large number of adipocytes, other non-fat cells, connective tissue matrix, vascular and neural tissues. The non-adipocytes cellular component includes inflammatory cells, immune cells, preadipocytes and fibroblasts (Ibrahim, 2010). The adipose tissue in pig is the privileged site of *de novo* lipid synthesis. Eighty percent of lipids are synthesised from dietary glucose, which is the main physiological precursor of fatty acids (Mourot & Hermier, 2001). Adipose tissues of industrial genotype pigs are principally SAT, which backfat accounts for at least 80% of the total adipose tissue of the carcasses. Backfat contains, approximately 75-80% lipids, 5-15% water and a little proportion of proteins as collagen. Lipids are mainly triacylglycerols (at least 99%) with a small amount of cholesterol and degradation products of triacylglycerols (Gandemer, 2002). The backfat represents the most important adipose component of the carcass. The backfat varies between breeds. The Iberian pigs and Alentejano purebred have a backfat very developed (23-30 mm thickness), while the commercial pig breeds have a backfat on average 15 mm thickness.

The fatty acid sums of backfat from commercial pigs are 36% of SFA, 44% of MUFA and 12% of PUFA (Davenel, Riaublanc, Marchal & Gandemer, 1999). In pigs at normal slaughter weight and fed a conventional diet, the approximate fatty acid composition of SAT is myristic (0-2%), palmitic (20-30%), palmitoleic (1-3%), stearic (10-15%), oleic (40-50%), linoleic (5-20%) and linolenic (1-5%) acids (Lizardo, Milgen, Mourot, Noblet & Bonneau, 2002). The amount of PUFA in adipose tissue influences the sensitivity to oxidative breakdown, and the formation of peroxides, and consequently the development of rancidity (Corino *et al.*, 2002). The fatty acid composition depends to numerous rearing factors, such as diet, breed, gender, age and physiological stage (Gandemer, 2002). Wood, Enser, Whittington, Moncrieff & Kempster (1989) reported that proportions of PUFA tend to be higher in SAT from entire males when compared to female pigs, and mainly due to their thinner backfat.

1.3. Adipogenesis and lipid metabolism

1.3.1. Adipogenesis

During the growth of the pig, lipid synthesis occurs firstly in SAT, then in intermuscular adipose tissues and finally in intramuscular adipose tissue, and this depends on the age of the animal (Lee & Kaufman, 1974). The muscle tissue consists mainly in myocytes, also known muscle cell or muscle fiber. Each myocyte contains myofibrils, which are very long chains of sarcomeres, the contractile units of the cell. Within the muscle cell, the myofibrils are bound together by perimysium into bundles called fascicles, the bundles are then grouped together to form muscle tissue. Myocytes and adipocytes are derived from a common mesenchymal precursor that has the potential to differentiate along several distinct lineages (Sordella, Jiang, Chen, Curto & Settleman, 2003). Preadipocytes and adipocytes are the important cells of adipose tissue. These are important in establishing the overall fatness of carcass, with being the main contributors to the marbling component required for consumer preference of meat products (Hausman *et al.*, 2009). Adipose depots in meat animals show differences at cellular, metabolic and genetic level. One example is subcutaneous porcine preadipocytes which proliferated more actively and showed more rapid accumulation of triacylglycerols than visceral-derived adipocytes (Samulin, Berget, Lien & Sundvold, 2008). Lin, Cross & Smith (1992) reported that intramuscular adipose tissue incorporates palmitic acid into storage lipids (triacylglycerols) at rates that exceed that SAT. Also, glucose contributes for a higher proportion of carbon to fatty acid biosynthesis in intramuscular than in SAT (Smith & Crouse, 1984).

Adipogenesis is the adipose tissue development that involves the proliferation of preadipocytes and their differentiation into mature adipocytes (Samulin, Berget, Grindflek, Lien & Sundvold, 2009). The proliferation of the stem cell population and the differentiation of these cells into adipocytes or vascular cells are the major processes involved in adipogenesis (Hausman, Digirolamo, Bartness, Hausman & Martin, 2001). Adipogenesis is of importance to animal production due to the implications of cost-effectively producing healthy animals that yield products with high consumer demand (Bergen & Mersmann, 2005). Adipogenesis is regulated by several key transcription factors, including CAAT/enhancer binding proteins (CEBPs) and peroxisome proliferator-activated receptor γ (PPARγ) (Hausman *et al.*, 2009). In adipose tissue, lipoprotein lipase (LPL) is an important marker for adipocyte differentiation, and their expression increases in parallel with cellular triacylglycerols accumulation as preadipocytes differentiate (Semenkovich, Wims, Noe, Etienne & Chan, 1989). Lipoportein lipase (LPL) is regulated at transcriptional, posttranscriptional, and posttranslational levels in a tissue-specific manner. LPL hydrolyze

triacylglycerol from triacylglycerol-rich lipoprotein particle to deliver fatty acids to different tissues. Thus, this enzyme is the key factor determining the dietary lipid deposition between tissues. LPL was predominantly expressed in mature adipocytes with a higher activity in subcutaneous adipose than those other kinds of adipose (Fielding & Frayn, 1998). LPL is synthesized in the parenchymal cells of heart, skeletal muscle and white and brown adipose tissues and spread along the vascular mesh. Following synthesis, LPL is secreted and then transported to the luminal surface of vascular endothelial cells (Wang & Eckel, 2009).

1.3.2. Lipid metabolism

Lipid metabolism includes the lipogenesis, or lipid synthesis, consisting on the synthesis of fatty acids and occurs in adipose tissue. In non-ruminants, such as pig, the principal site of fatty acid *de novo* synthesis is the adipose tissue and the glucose is their carbon precursor source. In adipose tissue, some processes determines fat deposition, which include the rates of fat uptake, *de novo* fatty acid synthesis, triacylglycerol synthesis, lipid degradation and transport processes of fatty acids (Hirsch & Han, 1969). The excess of glucose is used for *de novo* fatty acid synthesis and consequently, the triacylglycerol storage in adipose tissue depots. However, for the very low density lipoproteins (VLDL) synthesis in liver requires availability of fatty acid resulting from adipose triacylglycerol lipolysis (Dodson *et al.*, 2010).
In adipose tissue, cardiac and skeletal muscle and liver, the fatty acids released from chylomicrons by LPL. LPL is the rate limiting enzyme for the conversion of chylomicrons and VLDL into chylomicron remnants and low density lipoprotein (LDL) in tissues. Therefore, LPL controls triacylglycerol partitioning between adipose tissue and muscle, thereby increasing fattening or providing energy in the form of fatty acids for muscle energy metabolism (Hocquette, Graulet & Olivecrona, 1998). Thus, in pigs *de novo* lipid synthesis and lipoprotein synthesis are functionally and anatomically separated (Dodson *et al.*, 2010). In pigs glucose carbons enter fatty acids biosynthesis via malony-CoA production through the acetyl-CoA carboxylase reaction and then palmitate production through fatty acid synthase (Figure 1.9). After palmitate is synthesized, stearate is produced by a single two-carbon elongation and then oleate is generated by desaturation of stearate at the ninth carbon atom (Rule *et al.*, 1995) by delta-9 desaturase (SCD) that is responsible to synthesize MUFAs from SFA (Nakamura & Nara, 2004).
There are some factors that determine the rates of *de novo* fatty acid biosynthesis, fat uptake from blood, transport of fatty acids in adipocytes and lipid degradation. Acetyl-CoA carboxylase (ACACA) (Liu, Grant, Kim & Mills, 1994) and fatty acid synthase (FASN) (Clarke, 1993) are key lipogenic enzymes controlling the rates of SFA biosynthesis. In addition, fatty acid binding protein 4 (FABP4) is responsible for fatty acids transport in adipocytes

(Hocquette *et al.*, 2010). FABPs play a crucial role in intracellular fatty acid transport by binding and properly targeting long-chain fatty acids to their correct metabolic sites (Chmurzynska, 2006). FABPs bind fatty acids, mediate cellular fatty acid transport, compartmentalization of cellular fatty acids, modulation of the activity of the regulatory enzymes and protection of cellular enzymes and membranes from the potentially adverse effects of fatty acids, and lastly have the role in controlling muscle fatty acid metabolism (Cortright, Muoio & Dohm, 1997). There are different FABP subtypes with different functions. FABP1 expression was induced in proliferating cells, whereas FABP3, FABP4 and FABP5 expression increased through preadipocyte differentiation (Samulin *et al.*, 2008). FABP4 is involved in upholding the balance between lipogenesis and lipolysis in differentiating preadipocytes and its expression is highly induced during late adipogenesis (Cock, Houten, & Auwerx, 2004). There are other genes involved in the lipid metabolism, such as the responsive-element-binding protein (ChREBP or MLXIPL), which is a transcriptional regulator of lipogenic and glycolytic genes. Also, the insulin-regulated glucose transporter (GLUT4) is the major insulin-responsive glucose transporter and has a central role in systemic glucose metabolism (Herman *et al.*, 2012). Also this author reported that MLXIPL regulates fatty acid synthesis and glycolysis, is highly regulated by GLUT4 in adipose tissue and is a key determinant of systemic insulin sensitivity and glucose homeostasis. In addition, MLXIPL is required for the improved glucose homeostasis resulting from increased GLUT4 expression in adipose tissue. However, GLUT4-mediated glucose uptake induces MLXIPL, which activates *de novo* lipogenesis in adipose tissue. The MLXIPL is a major regulator of de novo lipogenesis enzymes in adipose tissue and mediates the changes in the enzyme expression resulting from increased glucose flux.

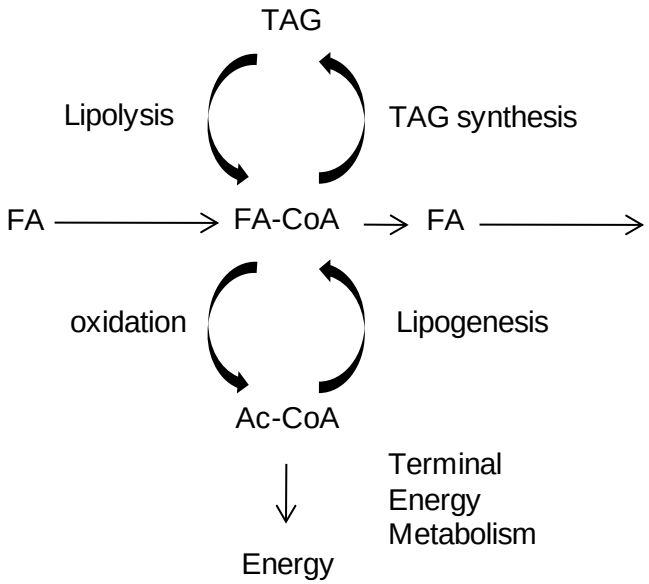

Figure 1.9 – A schematic diagram of major pathways of cellular fatty acid metabolism. Adapted from Wang *et al*. (2008). TAG, triacylglicerol; FA, fatty acids.

1.3.2.1. Stearoyl-CoA desaturase

Stearoyl-CoA desaturase (SCD) or delta-9 desaturase is an endoplasmatic reticulum-bound enzyme required for the biosynthesis of unsaturated fatty acids, which catalyses de 9-*cis* desaturation of saturated fatty acyl-CoAs. Is a rate-limiting enzyme in the cellular synthesis of monounsaturated fatty acids from saturated fatty acids (Ntambi, 1999) (Figure 1.10). It is located primarily in the endoplasmatic reticulum membrane and catalyses the oxidation of a spectrum of fatty acyl-CoA between carbons 9 and 10 with a preference for palmitoleoyl and stearoyl-CoA, wich are converted respectively into palmitoleoyl and oleoyl-CoA (Miyazaki & Ntambi, 2003; Ren, Knorr, Huang & Brenig, 2004). The principal products of SCD are the oleic acid and palmitoleic acid, which are the major fatty acids in fat depots and membrane phospholipids. The membrane fluidity and cell-cell interaction are significant influenced by the ratio of stearic acid to oleic acid incorporated into phospholipids (Ren *et al.*, 2004).
Stearoyl-CoA by ∆9-desaturase synthesizes the oleic acid and the activity of this enzyme could be an important determinant of the amount of stearic and oleic acids in porcine tissue lipids. As a result, ∆9-desaturase would be expected to be involved in pork meat quality (Kouba, Mourot & Peiniau, 1997). It is well know that in pigs, fatty acid synthesis occurs in adipose tissue that is also the principal site of stearoyl-CoA desaturation (Ho, Elliot. & Jone, 1975). In mice four isoforms of stearoyl-CoA desaturases have been characterized (Nakamura & Nara, 2004), and in cows, sheep, pigs and humans two isoforms were identified (Ren *et al*., 2004). The two isoforms that have been reported in pigs are SCD1, which is preferentially expressed in SAT (Ren *et al.*, 2004), and SCD5, which has been reported to be expressed at very high levels in brain and pancreas (Wang *et al.*, 2005). PUFA reduce the SCD expression, as well as the other lipogenic genes, by a repression of sterol regulatory element-binding proteins (SREBP1) activity. Moreover, PUFA activate peroxisome proliferator activated receptors (PPARs) to modulate the gene expression, in response to environmental stimuli (Miyazaki & Ntambi, 2003). Furthermore, SCD gene expression in liver and adipose tissue is regulated by different factors (Dobrzyn & Dobrzyn, 2006), which are dietary fat (PUFA, cholesterol and vitamin A), hormonal signals (insulin, glucagon), environment factors (temperature, metals, alcohol), peroxisomal proliferators and development processes (Ntambi & Miyazaki, 2004). Therefore Doran *et al*. (2006) reported that in pigs fed RPDs, there was an activation of SCD expression of the muscle but not in subcutaneous fat.

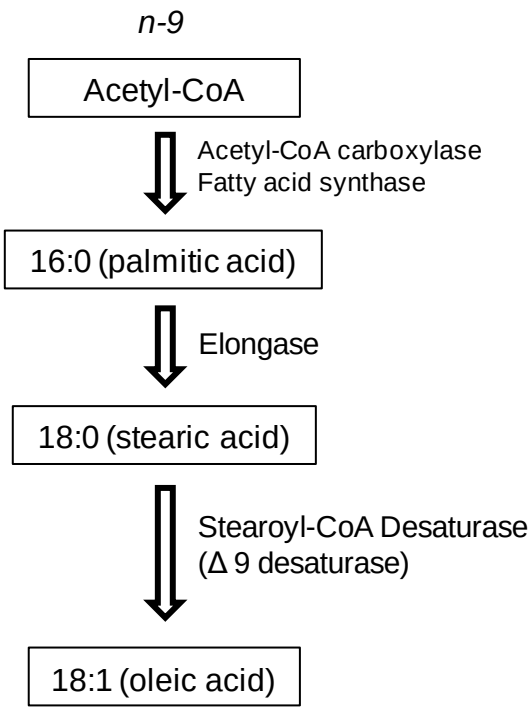

Figure 1.10 – Synthesis of monounsaturated fatty acids. Adapted from Nakamura & Nara (2004).

1.3.2.2. Delta-5 and Delta-6 desaturases

Delta desaturases create a double bond at a fixed position counted from the carboxyl end of fatty acids. Delta-5 and Delta-6 desaturases are required for the synthesis of long chain polyunsaturated fatty acids (LC-PUFA) (Figure 1.11), which are mainly esterified into phospholipids and contribute to maintaining membrane fluidity kingdoms. These desaturases are widely expressed in human tissues, with the highest levels in liver (Nakamura & Nara, 2004). In humans, Δ5 and Δ6 desaturase genes exhibit 75% sequence similarity and are located in the same region of chromosome 11. Fatty acid desaturase 1 gene (FADS1) encoding Δ-5 desaturase and fatty acid desaturase 2 gene (FADS2) encoding Δ-6 desaturase. The essential fatty acids, linoleic (18:2) and linolenic (18:3) are catalysed for desaturation by Δ6 desaturase for the production of LC-PUFA, such as arachidonic (20:4) and docosahexanoic (22:6, DHA) acids, with an introduction a double bond at position Δ6 on the acyl chain, which are required for various physiological functions for mammals including humans (Nakamura & Nara, 2004). The Δ5 desaturases is another front-end desaturase present in animals, that catalyses HUFA synthesis. The Δ5-desaturases introduces another double bond at the Δ5 position of 20-carbon fatty acids 20:3*n-6* and 20:4*n-3* (Figure 1.11), after desaturation and elongation by Δ6-desaturation and elongation, respectively. The enzymes for elongation and desaturation are found in the microsomal fraction cells.

The enzyme activity varies among organs of animals, with the highest activity in the liver and the adrenal glands and only limited activity in tissues such as the heart, kidneys and brain (Bézard, Blond, Bernard & Clouet, 1994).

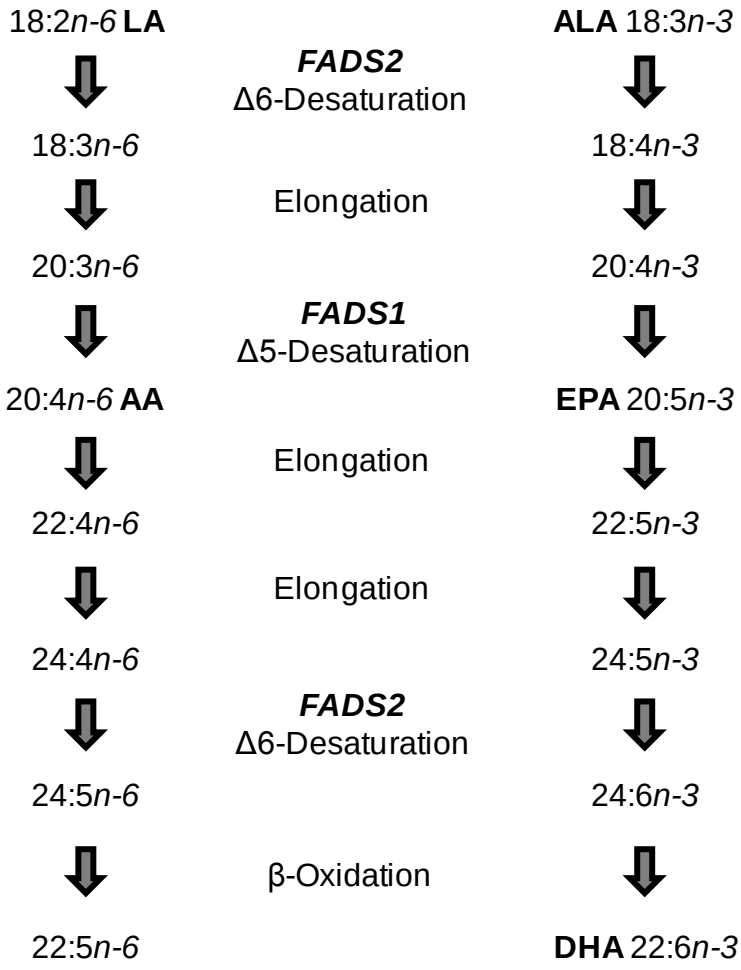

Figure 1.11 – Pathways for LC-PUFA synthesis from *n-6* (left) and *n-3* (right) essential fatty acids by enzymatic desaturation and chain elongation. Adapted from Glaser, Heinrich & Koletzko (2010).

1.3.3. Beta-oxidation of fatty acids

In most organisms fatty acid degradation occurs primarily via the β-oxidation cycle. β-oxidation or oxidation of fatty acids is an important metabolic process that occurs in various organisms, ranging from bacteria to higher eukaryotes. It is achieved in a series of four reactions that successively and repetitively cleave acetyl-coenzyme A fragments from fatty-acyl-CoA molecules (Kim & Battaile, 2002). In mammals, β-oxidation occurs in two distinct systems, mitochondrial and peroxisomal. Mitochondrial β-oxidation provides acetyl groups that can be degraded to CO_2 and H_2O for the production of ATP, and is strongly coupled to

the mitochondrial respiratory chain (Kim & Battaile, 2002). In contrast, β-oxidation in the peroxisome is involved in the metabolism of a variety of fatty acids, including branched-chain fatty acids and prostaglandins (Kim & Battaile, 2002). Quantitatively, β-oxidation is the major degradative pathway for fatty acids esters, which in animal cells takes place in both mitochondria and peroxisomes and thus is an example of metabolic compartmentalization (Poirier, Antonenkov, Glumoff & Hiltunen, 2006). Mitochondrial fatty acid β-oxidation generates acetyl-CoA and reducing equivalents (NADH and $FADH_2$), which are linked to the Krebs cycle and the mitochondria respiratory chain, leading to ATP production by oxidative phosphorylation in aerobic tissues (Sim, Hammond & Wilcken, 2002). There are some enzymes involved in β-oxidation step, such as carnitine O-acetyltransferase (CRAT), carnitine palmitoyltranferase 1 (CPT-1) and peroxisome proliferator-activated receptor alpha (PPARα). CRAT is the rate limiting enzyme of lipid catabolism, transporting fatty acid esters from cytosol to mitochondria for β-oxidation (Van der Leij, Huijkman, Boomsma, Kuipers & Bartelds, 2000), whereas PPARα is a major inducer of fatty acid oxidation (Poulsen, Siersbaek & Mandrup, 2012). CPT-1 is the enzyme that catalyses the rate-limiting step for the transport of acyl-CoA across the mitochondrial membrane and is widely considered the most important regulatory enzyme in determining the cellular rate of free fatty acid oxidation (Cortright *et al*., 1997). The activity of CPT-1 is inhibited by malonyl-CoA that is a precursor for *de novo* fatty acid synthesis and acts as the key regulator of acyl-CoA entry into mitochondria (Cortright *et al.*, 1997).

1.3.4. Regulation of lipid metabolism by transcription factors

It is well known that the transcription factors SREBP1, CCAAT/enhancer binding protein alpha (CEBPα) and PPARγ, are involved in the control of lipid metabolism in adipose tissue via regulation of expression of key enzymes and proteins controlling adipogenesis and lipogenesis (Kokta *et al*., 2004; Hocquette, Tesseraud, Cassar-Malek, Chiliard & Ortigues-Marty, 2007; Zhao *et al*., 2010).

In muscle, expression of lipogenic genes may be associated with the IMF content. The different PPARs can be considered key messengers responsible for the translation of nutritional, pharmacological and metabolic stimuli into changes in the expression of genes, more specifically those genes involved in lipid metabolism (Schoonjans, Staels & Auwerx, 1996). Peroxisomal enzymes are involved in a large range of catabolic and anabolic enzymatic pathways, such as β-oxidation of long-chain fatty acids, fatty acid elongation, the hydrolysis of acyl-CoAs and the conversion of acyl-CoAs to acylcarnitines, catabolism of purines and amino acids.

There are three types of PPAR, α, β and γ, each with a specific tissue distribution, compose a subfamily of the nuclear hormone receptor gene family. PPARα is involved in fatty acid oxidation by up-regulating the expression of the acyl-CoA oxidase and carnitine palmitoyltrasnferase enzymes (Torra, Gervois & Staels, 1999). In rodents, PPARα is highly expressed in tissue with considerable rates of fatty acid utilization such as liver, kidney and heart (Bell *et al*., 1998). PPARγ is DNA-binding transcription factor and has been implicated in fat metabolism and deposition through regulation of expression of various genes (Guo, Tang, Wang, Liu & Wang, 2011). Meadus, MacInnis & Dugan (2002) reported that differences in PPARγ expression between IMF and SAT may result in differences in fat deposition between the two fat locations. In adipocyte differentiation, the increase in lipolytic capacity which results from the induction of LPL by PPARγ will result in an increased delivery of fatty acids to the cells, this is enhanced by the PPAR-mediated induction of acyl-CoA synthetase (ACS) (Schoonjans *et al*., 1996). Thus, delivered fatty acids will provide the necessary building blocks for triglyceride synthesis and accumulation in the cell. Also, fatty acids and their metabolites are strong activators of PPAR. Schoonjans *et al.* (1996) suggested that PPARγ and its target genes play an interdependent role in adipocyte differentiation for the reason that fatty acids and fatty acid analogues induce the expression of adiposity-specific genes and enhance adipocyte conversion creating a positive feedback (Figure 1.12), which involves PPARγ and PPAR target genes, such as LPL, ACS and fatty acid transport protein (FATP), and which promotes and maintains the mature adipocyte phenotype.

PPARγ and C/EBPα are adipogenic transcription factors associating with lipid transport and metabolism, and expressed as later markers of differentiation during preadipocyte differentiated into fully mature adipocytes (Schoonjans *et al*., 1996). Sterol regulatory element-binding proteins (SREBPs) are transcription factors that regulate the expression of genes connected with cholesterol and fatty metabolism (Kersten, 2001). The SREBP1 induced the SCD expression and there are two isoforms of SREBP1 (SREBP-1a and -1c), that are involved in the regulation of genes of fatty acid biosynthesis (Paton & Ntambi, 2009).

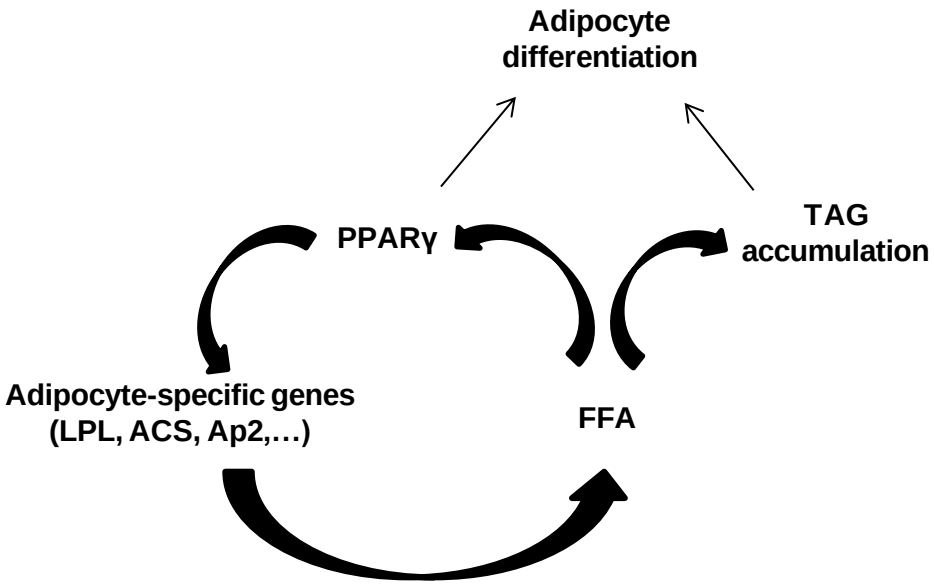

Figure 1.12 – Interdependent role of adipocyte-specif proteins such as LPL, ACS, FAT, FATP and PPARγ in adipocyte differentiation. Adapted from Schoonjans *et al.* (1996). LPL, lipoprotein lipase; ACS, acyl-CoA synthetase; FATP, fatty acid transport protein; Ap2, adipocyte fatty acid binding protein P2; FFA, free fatty acid; PPARγ, peroxisome proliferator activated receptor gamma; TAG, triacylglycerol.

1.4. Improved pork quality by nutritional regulation of lipid metabolism

Pork from modern lean lines of pigs may have poorer eating quality than other fatter genotypes, and this fact result in reduced consumer satisfaction, thus several strategies have been studied to improve pork quality. There are some factors affecting eating quality of pork, such as cooking, breed, gender and diet. Feeding strategies have been the most used to increase IMF and change fatty acid composition, and, consequently, to improve meat quality and sensory attributes for the consumer. Some of these strategies are the application of RPDs and amino acids supplementation.

1.4.1. Reduced protein diets

Several studies have been conducted in the last decades to reduce the amount of protein diets for pigs (Castell, Cliplef, Poste-Flynn & Butler, 1994; Goerl, Eilert, Mandigo, Chen & Miller, 1995; Kerr & Easter, 1995; Doran *et al*., 2006). Different dietary protein levels have been studied. Castell *et al*. (1994) in their study reported that different dietary protein levels (11.9%, 13.3%, 14.8%, 16.2% and 17.6%) increased marbling and IMF contents in *longissimus dorsi* muscle of pigs. Also Goerl *et al*. (1995) used different dietary protein levels in diets in pigs from 30-110 kg of body weight reported that in *longissimus lumborum* IMF

content increases with the decrease of protein in the diet. However Kerr & Easter (1995), in a study with low protein diets with and without amino acids supplementation reported that low protein diets without supplementation of amino acids, to pigs from weaning until market weight, increased IMF contents in *longissimus lumborum* muscle to the level of twice that pigs fed either high protein diets or low protein diets supplemented with lysine, tryptophan and threonine. Others studies with reduction of dietary protein from 20% to 16% also significantly increased IMF in growing pigs (Da Costa *et al*., 2004; Wood *et al*., 2004). Doran *et al*. (2006) reported that reduced protein level in diets increased total fatty acids in porcine muscle and this fact was related with higher activity of a key lipogenic enzyme, stearoyl-CoA desaturase, indicating that MUFA synthesis played a role when IMF accumulation was promoted by dietary low protein levels. In addition, Alonso, Campo, Provincial, Roncalés & Beltrán (2010) used the diets with 17% and 15% of crude protein and observed an increase in IMF content in muscle under RPD (15% of crude protein) but with similar dietary lysine content. Therefore, several studies showed that RPDs also changed the fatty acid composition in muscle. Wang *et al*. (2012) reported that the percentage of 16:0, 18:0 or SFA between low protein diet and high protein diet were similar, but the individual MUFA and total MUFA were higher in pigs fed low protein than in pigs fed high protein level, at all body weights. Moreover, Teye *et al.* (2006) and Alonso *et al.* (2010) reported that the content of PUFA was significantly lower with higher IMF in pigs fed low protein diets. The differences of fatty acid composition between pigs fed high and low protein diets may be due to the variance of SCD. The results obtained by Wang *et al.* (2012) indicated that low protein diet increased the deposition of IMF mainly by up-regulation of intramuscular lipogenic gene expression and down-regulation of lipolytic gene expression (Figure 1.13). Despite of the RPDs increase IMF and improve meat quality, some studies reported the negative effects, such as slower growth rate, thicker backfat and smaller *longissimus dorsi* muscle area (Goerl *et al*., 1995; Kerr *et al*., 1995).

1.4.2. Low dietary lysine levels

Reducing protein content in diets results in a reduction of all the amino acids. This is particularly important to the lysine content, which is the first limiting amino acid for muscle protein synthesis in pigs, i.e. the synthesis is limited if there is no available lysine for metabolism. However, it is not clear, if the increase of IMF is due to RPDs or low lysine levels in these diets. Previous research showed that IMF was increased in pigs fed lysine deficient diets (Cisneros *et al*., 1996; Blanchard *et al*., 1999; Witte, Ellis, McKeith & Wilson, 2000). It is well known that dietary lysine deficiency reduces protein synthesis and increases the amount of energy available for fat deposition (Witte, Ellis, McKeith & Wilson, 2000).

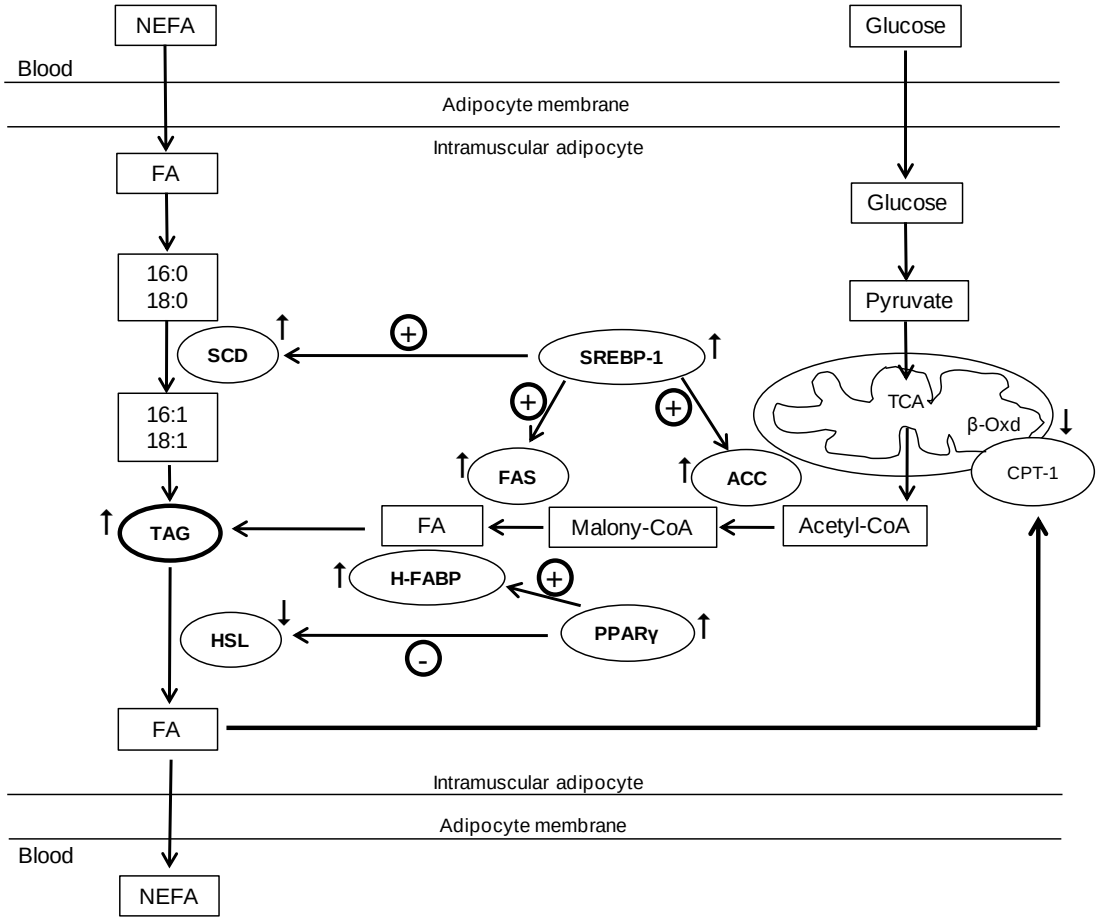

Figure 1.13 – Scheme of the impact of low dietary protein on lipid metabolism in porcine muscle tissue. ↑: Up-regulation of the gene expression or increased concentration of metabolite. ↓: Down-regulation of gene expression or decreased concentration of metabolite. +, Enhanced metabolic pathway; -, Inhibited metabolic pathway; NEFA, non-esterified fatty acid; FA, fatty acid; SCD, steroyl-CoA; TAG, triacylglycerols; HSL, hormone-sensitive lipase; SREBP-1, sterol regulatory element binding protein 1; FAS, fatty acid synthase; H-FABP, heart fatty-acid binding protein; ACC, acetyl-CoA carboxylase; PPARγ,peroxisome proliferator-activated receptor γ; TCA, tricarboxylic acid cycle; β-Oxd, β-oxidation; CPT-1, carnitine palmitoyltransferase-1. Adapted from Wang *et al.* (2012).

However, feeding lysine deficient diets is generally associated with reduced growth performance. The mechanisms associated to IMF accumulation in pigs fed deficient lysine diets are still unknown, but there are two possible mechanisms relevant to lipid metabolism. Katsumata, Matsumoto, Kobayashi & Kaji (2008) reported that in growing pigs fed low lysine diet, in the *longissimus dorsi* muscle there was high values of mRNA of *PPARγ*, *SREBP1* and fatty acid synthase. Therefore, PPARγ and SREBP1 are known to promote lipogenesis (Horton, 2002; Schadinger, Bucher, Schreiber & Farmer, 2005) and this suggesting that lipogenesis is enhanced in muscle due to dietary low lysine. Another possibility mechanism is the reduction of β-oxidation of fatty acids in muscle (Katsumata, Kyoya, Ishida, Ohtsuka & Nakashima, 2012). Katsumata, Kobayashi, Matsumoto, Tsuneishi & Kaji (2005) observed that free L-carnitine content in *longissimus lumborum* muscle was lower in finishing pigs fed

low lysine diet. While L-carnitine plays an essential role in β-oxidation of fatty acids by transporting fatty acyl-CoA across the internal mitochondrial membrane, then dietary low lysine possibly will suppress β-oxidation of fatty acids, consequently lipolysis in porcine muscle (Katsumata *et al.*, 2012). Katsumata (2011) reported that low dietary lysine levels does promote IMF accumulation, and that differentiation of adipocytes is unlikely to be involved in this response, as far as growing-finishing pigs are concerned.

1.4.3. Dietary supplementation with amino acids

Some studies have been developed using amino acids supplemented diets to improve pork quality. Leucine and arginine supplementation have been explored to improve meat quality traits. Some investigations showed that high dietary leucine levels increased IMF in late-finishing pigs (Cisneros *et al.*, 1996; Hyun *et al.*, 2003). Harris, Joshi & Jeoung (2004) reported that leucine plays an important role in protein synthesis, promotes insulin release, and inhibits protein degradation. Furthermore, leucine is a ketogenic amino acid, the carbon skeleton of which is converted to acetyl-CoA for fatty acid synthesis in muscle tissue. Hyun *et al.* (2003) obtained 2.4% of the average IMF content in *longissimus dorsi* muscle in the pigs fed the control diet, while obtained 3.4% in pigs fed a diet supplemented with leucine at the level of 2% with a body weight from 75 kg to 115 kg. However, the average of growth rate with this diet was approximately 100 g lower than that of the control pigs (829 g to 930 g, respectively). In another more recent study, Hyun *et al.* (2007) reported that the effects of leucine supplementation enhancing IMF content was observed only when leucine supplementation was combined with dietary low lysine, and not observed when dietary lysine was high. A study that investigated the effects of conjugated linoleic acid (CLA) and leucine and its combination on pork quality in finishing pigs concluded that supplementing leucine and CLA in finishing pig diets improved pork quality (IMF and tenderness), especially when 0.5% CLA was combined with 2.0% leucine (Yu *et al.*, 2007).

Arginine is another amino acid that has been investigated to as dietary supplement to improve meat quality of finishing pigs. Ma *et al*. (2010) using three levels of arginine (0, 0.5 and 1%) reported that arginine did not affected growth performance or carcass traits and that 1% of arginine increased IMF content. Thus this author concluded that supplemental arginine improved meat quality and attenuated oxidative stress of finishing pigs. However, Go *et al*. (2012) in a study with combination of CLA and arginine diets in growing pigs reported that IMF content increased with CLA supplementation, but not with arginine or the combination of both, while arginine increased backfat thickness in the absence or presence of CLA. Also, arginine supplementation had an effect on muscle fatty acid composition, Tan *et al.* (2011) reported that the percentage of oleic acid was higher but that of stearic and linoleic acid was

lower in muscle in pigs fed diet with arginine supplementation, when compared with the control pigs. This author, also demonstrate that dietary arginine supplementation up-regulates expression of lipogenic genes is skeletal muscle, while down-regulates expression of lipogenic genes and increases expression of lipolytic genes in white adipose tissue.

1.5. Objectives

The overall aim of the current study was to elucidate the effect of different feeding strategies to improve fat partitioning and meat quality in pigs and provide insights into genetic and metabolic mechanisms.

The specific aims of this study were as follows:

1. To investigate the combined effect of genotype (Alentejana purebred and a commercial crossbred pigs), dietary protein and lysine levels on pig growth, feed ingestion, carcass traits, meat quality and sensory attributes of pork (chapter 2).
2. To study the effects of RPDs with or without lysine adjustment on fatty acid composition and gene expression in *longissimus lumborum* muscle and subcutaneous adipose tissue of Alentejana purebred and Large White × Landrace × Pietrain crossbred pigs (chapter 3).
3. To evaluate the combined effect of arginine, dietary protein levels and leucine supplementation of commercial crossbred pigs on pig growth, feed ingestion, carcass traits, meat quality and sensory attributes of pork (chapter 4).
4. To assess the fatty acid composition and gene expression in *longissimus lumborum* muscle and subcutaneous adipose tissue of crossbred pigs fed RPDs with arginine and/or leucine supplementation (chapter 5).

Chapter 2

THE INCREASED INTRAMUSCULAR FAT PROMOTED BY DIETARY LYSINE RESTRICTION IN LEAN BUT NOT IN FATTY PIG GENOTYPES IMPROVES PORK SENSORY ATTRIBUTES

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Contribution of Marta S. Madeira to this article:
Marta S. Madeira collaborated in animal experiment, tissues sampling and did the meat quality parameters, laboratory analysis, data processing and statistical analysis. In addition, Marta S. Madeira integrated the trained sensory analysis panel and help in the interpretation of the results and writing of the manuscript.

[*] These co-authors contributed equally for this paper.

The increased intramuscular fat promoted by dietary lysine restriction in lean but not in fatty pig genotypes improves pork sensory attributes

Abstract

Sixty entire male pigs from 2 distinct genotypes (30 Alentejano purebred, an autochthonous fatty genotype, and 30 commercial crossbred pigs, a lean genotype) were used to investigate the effects of dietary crude protein reduction and low-lysine levels on growth performance, carcass traits, and meat quality. Pigs with 59.9 ± 2.0 kg body weight were randomly assigned within each genotype to 1 of 3 diets [(normal crude protein diet (control), reduced crude protein diet adjusted for lysine (RPDL), and reduced crude protein diet not adjusted for lysine, (RPD)] as a 2 × 3 factorial arrangement of treatments with 10 individually fed pigs per treatment. Pigs were slaughtered at 93.4 ± 2.4 kg body weight. The results showed that IMF content of *longissimus lumborum* muscle was greater in Alentejano than crossbred pigs (5.0 *vs*. 2.4%). The RPDL had no effect on IMF content, average daily gain (ADG), backfat thickness, and loin weight in both genotypes. The RPD promoted the increase (P<0.05) in IMF content in crossbred (about 50%), but not in Alentejano pigs, which indicates that lysine restriction can mediate the effect of RPD. Within crossbred pigs, meat obtained from pigs fed the RPD had an increased IMF content (+1.3%) and a tendency for greater sensory scores (tenderness, juiciness, flavour, and acceptability) than those fed the control. The IMF content was positively correlated to flavour in Alentejano genotype (P<0.05) but not in crossbred pigs. Alentejano and crossbred pigs had a greater tendency to deposit 18:1*c*9 and SFA, respectively. Despite the contribution of fatty acid composition to flavour, its influence on pork acceptability was more noticeable in crossbred than Alentejano pigs. In conclusion, the increased IMF promoted by dietary crude protein reduction in lean but not in fatty pig genotypes during the growing-finishing period is likely due to lysine limitation, which seems to enhance eating quality of pork.

Key words: crude protein, fatty acid, lipid, lysine, pork quality, sensory panel

2.1. Introduction

Intramuscular fat content is generally accepted as a major determinant of sensory traits of meat (Wood *et al.*, 2008). To reduce carcass fatness and improve feed efficiency, pigs reared in intensive systems have become leaner and faster growing. As a consequence, the eating quality of pork has been compromised because of its low IMF content. The IMF content positively influences meat sensory traits, such as taste and flavour (Damon *et al*., 2006; Hocquette *et al*., 2010). A decrease of IMF level below 2.5% has been shown to be related to lower pork sensory traits (Fernandez *et al*., 1999a). Therefore, the production of pork with moderate amounts of IMF, without an increase in subcutaneous fat, would be highly desirable for the meat industry.

Several studies indicated that dietary protein reduction during the growing-finishing period of pigs could enhance IMF content without increasing subcutaneous fat (Karlsson *et al*., 1993; Castell *et al.*, 1994). However, low-protein diets not adjusted for lysine content decreased the ADG and longissimus muscle area in commercial crossbred genotypes (Teye *et al*., 2006). Nonetheless, RPDs may increase IMF without affecting growth rate and protein deposition. Moreover, the effect of dietary protein and lysine levels on genotypes with distinct body fat content and protein deposition rate remains to be established.

In this investigation, we tested the hypothesis that RPDs, with or without an adjusted level of lysine, increases IMF content of lean commercial crossbred and fatty autochthonous purebred pigs, without major undesirable effects on growth rate and carcass traits. In addition, we postulated that increased IMF induced by RPDs improves meat sensory traits of lean genotype compared with fat pig genotype.

2.2. Material and Methods

This experiment was conducted in accordance with European Union standard guidelines for human care and use of animals in experimental research (Directive 86/609/EEC).

2.2.1. Animals and experimental diets

Sixty entire male pigs, 30 Alentejano purebred (an autochthonous high-fat porcine genotype) and 30 crossbred pigs with three genetics lines (50% Large White × 50% Landrace gilts mated to 50% Large White × 50% Pietrain boars; a commercial low-fat porcine genotype) with a body weight of 59.9 ± 2.0 kg were selected. Before the beginning of the experiment, all animals were housed and fed with the same conventional feed management (starter diet containing 13.0 MJ ME/kg and 1.2% total lysine from weaning to one month of age, followed

by an intermediate diet containing 13.0 MJ ME/kg and 1.1% total lysine until 25 to 30 kg body weight, and, then, a finishing diet containing 13.0 MJ ME/kg and 0.9% total lysine until about 60 kg body weight). Afterward, pigs were divided into groups of 10 animals, which were randomly allocated into 6 groups of 3 pens (2 pens containing 4 pigs and 1 pen containing 2 pigs) each and randomly assigned to 1 of the 3 diets within a 2 × 3 factorial arrangement (2 genotypes and 3 levels of crude protein and lysine) with individual control of feed intake. During the experiment, animals were individually fed twice a day and had ad libitum access to water. Feed offered and refusals of each pig were recorded daily to determine individual feed intake. Three isoenergetic diets (13.5 MJ ME/kg) were formulated to contain 0.60%, 0.60%, and 0.40% lysine for the control diet, reduced crude protein diet adjusted for lysine (RPDL), and reduced crude protein diet not adjusted for lysine (RPD), and L-Lysine was added to the RPDL diet to balance for lysine (Table 2.1). Analyzed contents of those diets were: 17.5% crude protein and 0.65% lysine for the control diet, 13.2% crude protein and 0.56% lysine for RPDL, and 13.1% crude protein and 0.40% lysine for RPD, and there was no difference in Lys content between the control and RPDL diets.

The dietary samples were collected 4 times during the experiment. Diets were analyzed for dry matter (DM) by drying a sample at 100 ºC to a constant weight. The nitrogen content was determined by the Kjeldahl method described in Association of Official Analytical Chemists (AOAC, 2000) and crude protein was calculated as 6.25 × nitrogen. The determinations of ash and starch contents were performed according to the procedures described by AOAC (2000) and Clegg (1956), respectively. Crude fat was determined by extracting feed samples with petroleum ether, using an automatic Soxhlet extractor (Gerhardt Analytical Systems, Königswinter, Germany). Crude fiber was determined according to the procedures described by AOAC (2000). The gross energy content of diets was measured using an adiabatic bomb calorimeter (Parr 1261, Parr Instrument Company, Moline, IL). Fatty acid methyl esters (FAME) of feed samples were analyzed by one-step extraction transesterification, using heptadecanoic acid (17:0) as internal standard (Sukhija & Palmquist, 1988). The amino acid composition was extracted according to the method described by AOAC (2005) and quantification was performed with HPLC (Agilent 1100, Agilent Technologies, Avondale, PA) according to Henderson, Ricker, Bidlingmeyer & Woodward (2000).

Table 2.1 - Ingredients and analyzed chemical composition of the experimental diets.

	Control	RPDL	RPD
Ingredients, %			
Barley	40.0	50.0	50.0
Wheat	26.1	29.5	29.0
Soybean meal	24.8	11.7	11.8
Corn	5.0	5.0	5.0
Soybean oil	1.6	1.5	1.5
Calcium carbonate	1.1	1.1	1.1
Vitamin-trace mineral premix[1]	0.4	0.4	0.4
Salt	0.4	0.4	0.4
Dicalcium phosphate	0.2	0.4	0.4
L-Lys	0.0	0.2	0.0
Phytase mixture[2]	0.1	0.1	0.1
Acid mixture[3]	0.1	0.1	0.1
Fermentation products[4]	0.1	0.1	0.1
Mold inhibitor mixture[5]	0.05	0.05	0.05
Antioxidant mixture[6]	0.003	0.003	0.003
Chemical composition			
DM, %	89.1	88.9	89.0
Crude protein, %	17.5[b]	13.2[a]	13.1[a]
Starch, %	47.2[a]	54.9[b]	55.2[b]
Crude fat, %	3.10	2.85	2.87
Crude fiber, %	4.87[b]	4.12[a]	3.97[a]
Ash, %	4.37[b]	3.92[a]	4.05[a]
Calcium, %	0.82	0.78	0.84
Phosphorus, %	0.37	0.37	0.37
Metabolisable energy (MJ ME/kg)	13.3	13.6	13.5
Lys:ME, %/MJ ME	0.049[b]	0.041[b]	0.030[a]
AA composition, %			
Ala	0.91[b]	0.63[a]	0.65[a]
Arg	1.32[b]	0.85[a]	0.88[a]
Asp	2.06[b]	1.29[a]	1.36[a]
Glu	4.62[b]	3.74[a]	3.68[a]
Gly	0.82[b]	0.55[a]	0.55[a]
His	0.51[b]	0.34[a]	0.35[a]
Iso	0.86[b]	0.57[a]	0.60[a]
Leu	1.58[b]	1.09[a]	1.14[a]
Lys[7]	0.65[b]	0.56[b]	0.40[a]
Met	0.12	0.11	0.10
Phe	1.07[b]	0.74[a]	0.78[a]
Pro	1.42[b]	1.19[a]	1.21[a]
Ser	1.00[b]	0.68[a]	0.70[a]
Tau	0.01	0.01	0.01
Thr	0.94[b]	0.69[a]	0.64[a]
Tyr	0.65[b]	0.45[a]	0.46[a]
Val	1.02[b]	0.73[a]	0.77[a]
Fatty acid composition, % total fatty acids			
14:0	0.14	0.15	0.16
16:0	17.3[a]	18.7[ab]	19.6[b]
16:1*c*9	0.16	0.18	0.17
18:0	2.59	2.56	2.57
18:1*c*9	19.0	18.9	19.3
18:1*c*11	1.54	1.52	1.56
18:2*n-6*	52.5	51.4	50.2
18:3*n-3*	4.93	4.61	4.30

20:0	0.31	0.31	0.33
20:1*c*11	0.42	0.45	0.48

[a,b]Within a row, means with different superscript letters differ, $P < 0.05$ ($n = 4$).
Control, normal crude protein diet; RPDL, reduced crude protein diet adjusted for Lys; and RPD, reduced crude protein diet without adjustment for Lys.
[1]Vita Tec (Tecadi, Santarém, Portugal). Provided per kilogram of diet: vitamin A, 6,000 IU; vitamin D3, 1,500 IU; vitamin E (acetate dl-alpha-tocopherol), 15 mg; vitamin B2, 0.3 mg; vitamin B12, 3.75 mg; biotin, 0.1 mg; calcium pantothenate, 12 mg; nicotinic acid, 15 mg; folic acid, 0.75 mg; choline chloride, 200 mg; Cu (cupric sulfate pentahydrate), 15 mg; Zn (zinc oxide), 100 mg; Mn (manganese oxide), 35 mg; I (potassium iodide), 0.7 mg; Co (basic cobaltous carbonate mono hydrous), 0.05 mg; Se (sodium selenite), 0.2 mg; Fe (ferrous carbonate), 80 mg; and BHT, 0.2 mg.
[2]3-phytase and calcium carbonate (Tecaphos 500 g; Tecadi).
[3]Formic acid, propionic acid, citric acid, and calcium salts (Ultracid V Dry EU; Tecadi).
[4]Fermentation product of *Aspergillus niger* fungus using wheat middlings as substrate combined calcium carbonate (Graintec TS; Tecadi).
[5]Hydrated aluminum silicates, Na, yeast extracts, calcium propionate, calcium formate, and antioxidant (Unike Plus Dry; Tecadi).
[6]Ethoxyquin, propyl gallate, and citric acid (Oxi-Nil Dry Premix; Tecadi).
[7]Calculated Lys content: 0.60, 0.60, and 0.40% for the Control, RPDL, and RPD, respectively.

2.2.2. Animal performance and muscle sampling

Throughout the experiment, pigs were weighed weekly before feeding. The ADG and gain:feed (G:F) were calculated. Feed was withdrawn from animals 17 to 19 h before slaughter. Pigs were slaughtered at a body weight of 93.4 ± 2.4 kg at the Unidade de Investigação em Produção Animal (UIPA-INIAV) experimental slaughterhouse, following standard handling procedures and using electrical stunning before exsanguination. The hot carcass weight (HCW) was recorded and carcass yield was calculated. Perirenal fat was removed and weighed. For IMF content and fatty acid composition, samples of *longissimus lumborum* muscle were collected from the right carcass side between third and fifth lumbar vertebras, immediately vacuum-packed and stored at -20 ºC until analyses.

At 24 h postmortem, backfat thickness was measured in the left carcass side at shoulder, last rib position (P2; the most representative location), last lumbar vertebra (L6), and second sacral vertebra (S2), as described by Frederick (1972). The loin was excised from the left carcass side between last cervical and L6 lumbar vertebras, and weighed just before being sliced into 2.5-cm-thick chops for sensory evaluation (second to third lumbar vertebras) and shear force measurements (first lumbar vertebra). Chops were vacuum packaged, frozen, and stored at -20 ºC, until further analyses.

2.2.3. Meat quality traits

The pH and temperature were measured in the *longissimus lumborum* muscle (L1, right carcass side) at 45 min (pH_{45} and T_{45}, respectively) and 24 h (pH_{24} and T_{24}, respectively) postmortem, using a pH meter equipped with a penetrating electrode (HI8424, Hanna Instruments, Smithfield, RI). Objective colour was measured on the cut surface of the longissimus muscle section 24 h postmortem, using a chromometer (Minolta CR-300; Konica Minolta, Tokyo, Japan) with 2° viewing angle geometry, 11 mm-diameter aperture, 8 mm-

diameter measurement area, and a C light source. The colour system was the CIE L* (lightness), a*(redness), and b* (yellowness), and the data were obtained 1 h after air exposure to allow blooming.

2.2.4. Intramuscular fat content and fatty acid composition

The IMF content was determined in fresh samples by hydrolysis with 4 M HCl, followed by Soxhlet extraction with petroleum ether as described by AOAC (2000). To assess fatty acid composition in muscle, total lipids were extracted from lyophilized samples (about 250 mg) using dichloromethane:methanol (2:1, v/v), the method adapted from Folch & Stanley (1957). Fatty acids were converted to FAME by a combined transesterification procedure with NaOH in anhydrous methanol (0.5 M), followed by HCl:methanol (1:1 v/v), at 50 °C during 30 and 10 min, respectively, according to the method described by Raes, De Smet & Demeyer (2001). The FAME were analyzed using a gas chromatograph (HP6890A; Hewlett-Packard, Avondale, PA) equipped with a flame ionization detector and a CP-Sil 88-capillary column (100 m × 0.25 mm i.d.; 0.20 mm film thickness; Chrompack, Varian Inc., Walnut Creek, CA). The chromatographic conditions were as follows: the injector and detector temperature were 250 and 280 ºC, respectively, helium was used as carrier gas, and the split ratio was 1:70. The gas chromatograph oven temperature was programmed to start at 100 ºC (maintained for 1 min) followed by a 50 ºC/min ramp to 150 ºC (maintained for 1 min), followed by a 2 ºC/min ramp to 200 ºC (maintained for 2 min), and finally increased at 30 ºC/min to 220 ºC (maintained for 15 min).

The quantification of total FAME was done using nonadecanoic acid (19:0) as internal standard and the conversion of relative peak areas into weight percentages. Fatty acids were identified on the basis of their retention times, corresponding to their FAME standards (Supelco Inc., Bellefonte, PA), and expressed as mg/100 g of muscle.

2.2.5. Shear force measurements

Frozen chops were thawed at 4 ºC (24 h) and cooked in a plate grill (65/70 FTES Electric Griddle, Modelar Catering Equipment, Italy) at 250 °C until they reached an internal temperature of 71 °C, which was monitored by an internal thermocouple (Lufft C120, München, Germany). The cooking loss was determined by calculating the difference in weight before and after thermal processing. One hour after cooling, 8 to 10 cores parallel to muscle fiber direction (1 cm^2) were taken from each steak. The Warner-Bratzler shear force (WBSF, kg) was measured with a texture analyzer (TA-tx2i Texture Analyser, Stable Micro Systems, Surrey, UK), equipped with a Warner-Bratzler shear device with a 30-kg compression load cell and a crosshead speed of 5 mm/s. Data were collected with specific

software (Texture Expert Exceed, Stable Micro Systems, Surrey, UK). The peak shear force measurements of cores from each steak were recorded and averaged to obtain a single WBSF value for each steak.

2.2.6. Trained sensory panel analysis

For each session of trained sensory panel analysis, 10 chops were thawed and cooked, using the same conditions described for shear force measurements. All samples were trimmed of external connective tissue and cut into cores with approximately 2 × 2 × 2 cm, maintained at 60 ºC in heated plaques and tasted as soon as possible. Twelve trained panelists of pork performed the sensory analysis in 6 sessions (10 samples for each session). The panelists were selected and trained according to Cross, Moen & Stanfield (1978). Samples were randomly distributed across sessions and the attributes classified were tenderness (defined as the opposite of the force required to bite through the sample with the molars), juiciness (amount of liquid drained from the sample during the initial chews), flavour (intensity with which the pork sample is recognized as distinctly swine meat, rather than any other species of meat), and overall acceptability (perception of how the meat is palatable, taking into account the aforementioned attributes). The scale applied in the sensory analysis was structured into 8 points, with 1 being extremely though, dry, weak, or negative, and 8 being extremely tender, juicy, strong, or positive for tenderness, juiciness, flavour, and overall acceptability, respectively.

2.2.7. Statistical analysis

Data were analyzed using PROC MIXED (SAS Inst. Inc., Cary, NC), with variance heterogeneity analysis and considering animal as the experimental unit, with genotype, diet, and their respective interaction as fixed effects. Fatty acids with contents greater than 20 mg/100 g muscle were included in the model. If the treatments were different (P<0.05 and tendencies discussed at P<0.10), means for traits were compared using the PDIFF option of SAS. The body weight at slaughter was included in the model as a covariate to adjust the carcass characteristics presented in Table 2.2. Pearson's correlation coefficients were calculated with the CORR procedure of SAS to elucidate possible associations among carcass characteristics, meat traits, and major fatty acid (expressed as g/100 g of total FA). The correlations were considered significant at P<0.05.

2.3. Results

2.3.1. Growth performance and carcass traits

The results of growth performance and carcass traits are shown in Table 2.2. The Alentejano pigs had average daily feed intake (ADFI) values greater and G:F values less than crossbred pigs (P<0.001). The genotype strongly affected (P<0.001) all carcass traits assessed, in contrast to diets, which only influenced (P<0.05) perirenal fat. On average, Alentejano breed pigs, when compared with crossbred genotype pigs, had less HCW, carcass yield, and loin weight (P<0.001). In contrast, perirenal fat and thickness of subcutaneous fat (assessed at shoulder, P2, L6, and S2 sites) were greater in Alentejano breed than in crossbred genotype (P<0.001). A tendency for a diet effect (P=0.051) on loin weight was obtained, with lower values for pigs of both genotypes fed RPD. In addition, reduced crude protein diets (RPDL and RPD) increased (P<0.05) the ADFI about 0.2 kg/d when compared with the control diet. Furthermore, G:F was reduced (P<0.05) in pigs fed the reduced crude protein diets, and it was less in RPD than RPDL (P<0.05). The perirenal fat was influenced by dietary crude protein level (P<0.05) but not by lysine content (P>0.05). A genotype × diet interaction (P=0.047) was found for ADG and crossbred pigs fed RPD had a lower ADG than crossbred pigs fed the control and RPDL diets (approximately 50 g/d), which was not observed for Alentejano pigs fed the same diet.

2.3.2. Meat quality traits

Meat traits in the *longissimus lumborum* muscle are presented in Table 2.3. Forty-five min after slaughter, T_{45} was greater (P<0.05) in Alentejano than in crossbred genotype. Despite the pH_{45} being increased 0.10 unit (P<0.01) in Alentejano pigs, the pH had a normal fall (above 6.0 at 45 min after slaughter), ultimately reaching pH_{24} of about 5.7 in both pig genotypes. This is compatible with the absence of unacceptable pale, soft, and exudative pork. Regarding colour measurements, meat from Alentejano pigs had less L* (P<0.001) and greater a* (P<0.01) than that from crossbred pigs. Compared with Alentejano pork, WBSF was around 1.0 kg greater (P<0.01) in meat obtained from crossbred pigs. There were no differences among dietary treatments on meat quality traits.

Table 2.2 - Growth performance and carcass characteristics of Alentejano and crossbred pig genotypes.

Item	Genotype		Diet			Significance level	
	Alentejano (n = 30)	Crossbred (n = 30)	Control (n = 20)	RPDL (n = 20)	RPD (n = 20)	Genotype	Diet
Growth performance							
ADFI, kg	3.24 ± 0.05	2.61 ± 0.05	2.78 ± 0.07^{b}	2.97 ± 0.05^{a}	3.04 ± 0.07^{a}	<0.001	0.025
ADG[1], g	801 ± 18	822 ± 20	829 ± 27^{a}	829 ± 25^{a}	776 ± 16^{b}	0.438	0.116
G:F, kg/kg	0.25 ± 0.01	0.31 ± 0.01	0.30 ± 0.01^{c}	0.28 ± 0.01^{b}	0.26 ± 0.01^{a}	<0.001	<0.001
Carcass characteristics[2]							
HCW, kg	73.0 ± 0.3	76.3 ± 0.2	74.7 ± 0.3	74.9 ± 0.3	74.4 ± 0.3	<0.001	0.597
Carcass yield, %	78.1 ± 0.3	81.7 ± 0.3	79.9 ± 0.3	80.2 ± 0.4	79.7 ± 0.3	<0.001	0.606
Perirenal fat, kg	2.01 ± 0.05	0.75 ± 0.02	1.28 ± 0.04^{b}	1.44 ± 0.04^{a}	1.41 ± 0.05^{ab}	<0.001	0.027
Shoulder, mm	45.4 ± 0.6	27.6 ± 1.5	34.9 ± 1.4	37.9 ± 1.5	36.6 ± 1.2	<0.001	0.371
P_2 backfat thickness, mm	29.9 ± 0.9	15.1 ± 0.8	21.4 ± 0.8	23.3 ± 1.2	22.8 ± 1.1	<0.001	0.346
L6 backfat thickness, mm	36.9 ± 1.0	19.8 ± 0.8	26.5 ± 0.8	29.3 ± 1.2	29.2 ± 1.3	<0.001	0.091
S2 backfat thickness, mm	29.6 ± 0.9	10.2 ± 0.5	19.1 ± 0.7	20.6 ± 1.0	20.1 ± 1.1	<0.001	0.429
Loin weight, kg	4.29 ± 0.06	5.53 ± 0.05	5.02 ± 0.07	4.93 ±0.07	4.77 ± 0.07	<0.001	0.051

[a-c]Within a row, means with different superscript letters differ, $P < 0.05$.
Control, normal crude protein diet; RPDL, reduced crude protein diet adjusted for Lys; RPD, reduced crude protein diet without adjustment for Lys.
[1]Genotype x diet interaction, $P = 0.047$. Interactive means : 809 ± 39, 788 ± 29, 806 ± 24, 848 ± 37, 871 ± 41, and 746 ± 23 for Alentejano pigs fed control, RPDL, and RPD, and crossbred pigs fed control, RPDL, and RPD, respectively.
[2]P2, at the last rib, L6, at the last lumbar vertebra, and S2, second sacral vertebra.

Table 2.3 - Meat traits of *longissimus lumborum* muscle from Alentejano and crossbred pig genotypes.

	Genotype		Diet			Significance level	
	Alentejano (n = 30)	Crossbred (n = 30)	Control (n = 20)	RPDL (n = 20)	RPD (n = 20)	Genotype	Diet
Temperature, ºC							
45 min	30.9 ± 0.2	30.2 ± 0.2	30.5 ± 0.2	30.4 ± 0.2	30.8 ± 0.3	0.013	0.545
24 h	9.49 ± 0.27	8.81 ± 0.32	9.30 ± 0.41	9.15 ± 0.35	8.99 ± 0.34	0.112	0.846
pH							
45 min	6.45 ± 0.03	6.35 ± 0.01	6.40 ± 0.03	6.39 ± 0.03	6.41 ± 0.03	0.009	0.847
24 h	5.73 ± 0.01	5.71 ± 0.02	5.70 ± 0.02	5.72 ± 0.01	5.72 ± 0.01	0.347	0.735
Colour measurements							
L*	50.0 ± 0.8	55.0 ± 1.0	52.7 ± 1.0	52.5 ± 1.2	52.3 ± 1.1	<0.001	0.977
a*	9.65 ± 0.58	7.49 ± 0.42	8.25 ± 0.63	8.54 ± 0.63	8.92 ± 0.59	0.004	0.741
b*	4.63 ± 0.32	4.19 ± 0.28	4.36 ± 0.40	4.26 ± 0.30	4.61 ± 0.41	0.315	0.791
WBSF, kg	5.93 ± 0.22	6.97 ± 0.30	6.60 ± 0.36	6.51 ± 0.24	6.25 ± 0.34	0.007	0.758
CL, %	14.2 ± 0.7	15.2 ± 0.7	14.9 ± 0.9	15.2 ± 0.8	14.0 ± 0.9	0.303	0.584

Control, normal crude protein diet; RPDL, reduced crude protein diet adjusted for Lys; and RPD, reduced crude protein diet without adjustment for Lys.
WBSF, Warner-Bratzler shear force; CL, cooking loss.

2.3.3. Intramuscular fat content and composition

The IMF content and fatty acid composition in *longissimus lumborum* muscle of Alentejano and crossbred pig genotypes are shown in Table 2.4. An interaction between genotype and diet ($P<0.05$) was observed for IMF, with no dietary effect for Alentejano pigs but with an increased IMF of 40% for crossbred pigs fed RPD. In contrast, RPDL did not ($P>0.05$) increase IMF in either Alentejano or crossbred pigs. Regarding major individual FA, genotype × diet interactions ($P<0.05$) were found for 16:0, 16:1*c*9, 18:0, and 18:1*c*9, with greater contents of those fatty acids in crossbred pigs fed RPD, compared with other crossbred pigs. When compared with crossbred pigs, Alentejano pigs had greater content ($P<0.01$) of 18:2*n-6* (linoleic acid, 178 *vs*. 159 mg/100 g muscle) and *n-3* PUFA (9.82 *vs*. 7.57 mg/100 g muscle) but less content ($P<0.001$) of 20:4*n-6* (arachidonic acid; 26.1 *vs*. 32.1 mg/100 g muscle). Interactions between genotype and diet ($P<0.05$) were observed for SFA and MUFA, with greater values in crossbred pigs fed RPD relative to other crossbred pigs. Compared with genotype, diets had only a small effect on individual fatty acids and partial sums of FA. Neither genotype nor diet influenced ($P>0.05$) total PUFA and *n-6* PUFA contents.

2.3.4. Trained sensory panel analysis

The trained sensory panel scores for the *longissimus lumborum* muscle of Alentejano and crossbred pig genotypes are presented in Table 2.5. Alentejano pigs had greater ($P<0.05$) tenderness, juiciness, and flavour scores, compared with crossbred pigs. A similar tendency ($P=0.055$) was observed for overall acceptability. Dietary treatment had only a small effect on trained sensory panel scores. However, meat from pigs fed RPD had greater ($P<0.05$) juiciness than that from control pigs. The same trend ($P=0.076$) was observed for overall acceptability scores.

Table 2.4 - Intramuscular fat content (IMF; g/100 g muscle) and fatty acid (FA) composition (mg/100 g muscle) of *longissimus lumborum* muscle from Alentejano and crossbred pig genotypes.

Item	Alentejano			Crossbred			Significance level		
	Control (n = 10)	RPDL (n = 10)	RPD (n = 10)	Control (n = 10)	RPDL (n = 10)	RPD (n = 10)	Genotype	Diet	Genotype × diet
IMF	4.16 ± 0.36^{b}	5.79 ± 0.92^{b}	4.47 ± 0.39^{b}	2.68 ± 0.28^{a}	2.16 ± 0.16^{a}	3.74 ± 0.35^{b}	<0.001	0.143	0.037
Major individual FA									
16:0	542 ± 43^{bc}	803 ± 96^{d}	628 ± 64^{cd}	353 ± 32^{a}	312 ± 24^{a}	462 ± 35^{b}	<0.001	0.050	0.027
16:1*c*9	59 ± 5^{bc}	93 ± 10^{d}	75 ± 6^{cd}	48 ± 6^{ab}	39 ± 5^{a}	59 ± 5^{bc}	<0.001	0.056	0.010
18:0	287 ± 23^{bc}	417 ± 51^{d}	321 ± 33^{cd}	174 ± 13^{a}	164 ± 11^{a}	236 ± 19^{b}	<0.001	0.049	0.040
18:1*c*9	801 ± 67^{b}	1,192 ± 155^{c}	910 ± 106^{bc}	519 ± 48^{a}	450 ± 38^{a}	695 ± 57^{b}	<0.001	0.078	0.031
18:2*n-6*	176 ± 7	194 ± 12	165 ± 7	159 ± 7	152 ± 4	165 ± 8	0.004	0.599	0.052
20:4*n-6*	28 ± 1	24 ± 1	26 ± 1	32 ± 2	32 ± 3	32 ± 2	<0.001	0.460	0.574
Partial sum of FA									
SFA	868 ± 68^{bc}	1276 ± 154^{d}	994 ± 101^{cd}	558 ± 47^{a}	502 ± 37^{a}	734 ± 55^{b}	<0.001	0.051	0.030
MUFA	998 ± 82^{b}	1,492 ± 188^{c}	1,148 ± 130^{bc}	678 ± 67^{a}	572 ± 49^{a}	885 ± 72^{b}	<0.001	0.083	0.024
PUFA	227 ± 9	245 ± 15	212 ± 8	214 ± 9	205 ± 6	221 ± 10	0.078	0.688	0.072
n-3 PUFA	8.7 ± 0.6	11.8 ± 1.4	8.9 ± 0.8	7.3 ± 0.5	6.9 ± 0.3	8.4 ± 0.6	0.002	0.234	0.053
n-6 PUFA	219 ± 9	234 ± 14	203 ± 7	207 ± 9	198 ± 6	213 ± 9	0.110	0.680	0.079

[a-c]Within a row, means with different superscript letters differ, $P<0.05$.
Control, normal crude protein diet; RPDL, reduced crude protein diet adjusted for Lys; and RPD, reduced crude protein diet without adjustment for Lys.
SFA = 12:0 + 14:0 + 16:0 + 17:0 + 18:0 + 20:0; MUFA = 16:1*c*7 + 16:1*c*9 + 18:1t + 18:1*c*11 + 20:1*c*11; PUFA = 18:2*n-6* + 18:3*n-3* + 20:2*n-6* + 20:3*n-6* + 20:3*n-3* + 20:4*n-6* + 22:4*n-6*; *n-3* PUFA = 18:3*n-3* + 20:3*n-3*; and *n-6* PUFA = 18:2*n-6* + 20:2*n-6* + 20:3*n-6* +20:4*n-6* + 22:4*n-6*.

Table 2.5 - Sensory panel scores of *longissimus lumborum* muscle from Alentejano and crossbred pig genotypes.

Item	Genotype		Diet			Significance level	
	Alentejano (n = 30)	Crossbred (n = 28)	Control (n = 20)	RPDL (n = 18)	RPD (n = 20)	Genotype	Diet
Tenderness	5.72 ± 0.08	5.47 ± 0.08	5.45 ± 0.09	5.63 ± 0.10	5.70 ± 0.09	0.028	0.164
Juiciness	4.17 ± 0.08	3.89 ± 0.08	3.86 ± 0.10^{b}	4.00 ± 0.10^{ab}	4.22 ± 0.10^{a}	0.016	0.043
Flavour	4.94 ± 0.07	4.69 ± 0.07	4.80 ± 0.09	4.69 ± 0.09	4.95 ± 0.09	0.013	0.120
Overall acceptability	5.16 ± 0.07	4.96 ± 0.07	4.92 ± 0.09	5.03 ± 0.09	5.21 ± 0.09	0.055	0.076

[a-c]Within a row, means with different superscript letters differ, $P < 0.05$.
Control, normal crude protein diet; RPDL, reduced crude protein diet adjusted for Lys; and RPD, reduced crude protein diet without adjustment for Lys.

2.3.5. Correlation between carcass parameters and meat quality

To avoid genotype confounding effects, correlations among meat traits were assessed separately for Alentejano and crossbred pigs. Correlation coefficients among IMF, FA, ADFI, ADG, G:F, HCW, carcass yield, loin weight, pH_{24}, WBSF, cooking loss, and trained sensory panel scores are shown in Table 2.6.

For the Alentejano genotype, as IMF increases, its composition changes (P<0.001) by increasing the proportion of 18:1*c*9 and decreasing the percentages of 18:2*n-6* and 20:4*n-6* fatty acids. Likewise, these relationships were also observed in crossbred pigs. However, the IMF deposition was associated with an increment of 16:0 and 16:1*c*9 proportions in the latter genotype. In addition, IMF deposition was positively and moderately correlated (0.7≥r≥0.3) with G:F (P<0.01) in both genotypes. Moreover, in crossbred pigs, the greater the loin weight, the less its IMF content. In contrast, moderate correlations between IMF and ADG (r=-0.37) and carcass yield (r=0.37) were observed in Alentejano pigs.

Several correlations among growth performance and carcass traits were recorded in both genotypes. In general, these correlations followed the same trend in both genotypes. However, HCW was directly correlated with ADG in crossbred genotype, but this relationship was not statistically significant (P>0.05) in Alentejano pigs. In contrast, carcass yield was negatively correlated with ADG, but this relationship was only statistically significant (P<0.05) in Alentejano genotype. Moreover, pH_{24} values showed small r with all trained sensory panel scores, except with tenderness in crossbred pigs (r=-0.37). In addition, tenderness and juiciness had a greater contribution than flavour to acceptability scores in pork from both genotypes. Indeed, moderate to high (r>0.70) correlations were found between acceptability and tenderness (r=0.90 and r=0.83) and juiciness (r=0.70 and r=0.52) in pork from Alentejano and crossbred genotypes, respectively. The latter meat sensory attribute was moderately correlated with flavour (r=0.66) in crossbred but not in Alentejano pigs.

Fatty acid profile had an important role in meat flavour scores in both pig genotypes and was related with meat acceptability values in the crossbred genotype. The increase of 18:1*c*9 was associated with greater flavour scores (P<0.001) in Alentejano pigs and greater acceptability scores (P<0.05) in crossbred pigs. In contrast, as 18:2*n-6* and 20:4*n-6* increased, lower meat flavour and acceptability scores were obtained in Alentejano and crossbred pigs, respectively. Moreover, the proportion of 20:4*n-6* was negatively correlated with acceptability but only in the crossbred pigs (r=-0.40). Finally, a moderate negative correlation between WBSF and tenderness (r=-0.43) was found for the meat from crossbred pigs. Nevertheless, excluding tenderness in meat from crossbred pigs, small relationships were observed between trained sensory panel scores and WBSF or cooking loss values.

Table 2.6 - Pearson´s correlation coefficients among intramuscular fat content (IMF, g/100 g muscle), major fatty acids (g/100 g total fatty acids), ADFI (kg/d), ADG, kg/d), G:F (kg/kg), HCW (kg), carcass yield (%), loin weight (kg), pH at 24 h, Warner-Bratzler shear force (WBSF, kg), cooking loss (%), and sensory panel scores of *longissimus lumborum* muscle from Alentejano and crossbred pig genotypes.

	Item	16:0	16:1*c*9	18:0	18:1*c*9	18:2*n*-6	20:4*n*-6	IMF	ADFI	ADG	G:F	HCW	Carcass yield	Loin weight	pH_{24}	WBSF	Cooking loss	Tendern.	Juiciness	Flavour	Acceptab.
Alentejano pig genotype	IMF	0.33	0.03	0.20	0.69***	-0.63***	-0.62***	1.00													
	ADFI	0.51**	0.32	0.29	0.01	-0.26	-0.25	0.05	1.00												
	ADG	0.05	0.05	-0.01	-0.27	0.26	0.19	-0.37*	0.44*	1.00											
	G:F	0.38*	0.17	0.25	0.33	-0.50**	-0.44*	0.47**	0.36*	-0.67***	1.00										
	HCW	-0.25	0.04	-0.29	-0.28	0.33	0.36	-0.01	0.01	-0.06	0.06	1.00									
	Carcass yield	0.11	0.01	-0.08	0.16	-0.19	-0.16	0.37*	-0.17	-0.41*	0.31	0.50**	1.00								
	Loin weight	-0.34	-0.21	-0.21	-0.25	0.35	0.22	-0.14	-0.27	0.03	-0.27	0.43*	0.12	1.00							
	pH_{24}	0.26	0.33	-0.24	0.23	-0.27	-0.20	-0.01	0.20	0.06	0.21	-0.11	0.11	-0.26	1.00						
	WBSF	0.01	-0.11	0.25	0.06	-0.12	-0.08	0.06	-0.00	0.01	-0.00	0.00	0.15	-0.23	-0.07	1.00					
	Cooking loss	-0.07	-0.11	0.11	0.11	-0.11	-0.09	0.22	-0.16	-0.19	0.09	-0.04	0.31	-0.11	-0.06	0.72***	1.00				
	Tenderness	-0.02	0.07	0.06	0.05	-0.01	0.01	0.24	0.12	0.21	-0.12	-0.06	0.01	-0.05	-0.10	-0.21	-0.19	1.00			
	Juiciness	0.18	-0.11	0.26	-0.04	-0.05	-0.09	0.17	0.16	0.20	-0.07	0.03	0.21	0.17	-0.08	-0.12	-0.05	-0.05	1.00		
	Flavour	0.19	0.05	-0.11	0.64***	-0.50**	-0.54**	0.44*	-0.24	-0.39*	0.23	-0.07	0.36	-0.04	-0.14	0.01	0.15	0.15	0.10	1.00	
	Acceptability	0.09	0.07	0.26	-0.06	-0.03	-0.03	0.14	0.23	0.23	-0.07	-0.10	-0.07	0.00	-0.13	-0.32	-0.26	0.90***	0.70***	-0.12	1.00
Crossbred pig genotype	IMF	0.65***	0.60***	0.01	0.57**	-0.75***	-0.65***	1.00													
	ADFI	0.34	0.03	0.19	0.33	-0.34	-0.29	0.13	1.00												
	ADG	0.11	-0.04	0.02	-0.14	0.10	0.01	-0.20	0.56**	1.00											
	G:F	0.19	0.08	0.13	0.50**	-0.45*	-0.28	0.38*	0.28	-0.63***	1.00										
	HCW	-0.09	-0.20	0.09	-0.12	0.14	0.20	-0.26	0.28	0.42*	-0.21	1.00									
	Carcass yield	-0.12	-0.07	-0.05	0.01	0.08	0.05	-0.11	-0.18	-0.03	-0.12	0.55**	1.00								
	Loin weight	-0.43*	-0.20	-0.35	-0.33	0.42*	0.50**	-0.42*	-0.06	0.16	-0.21	0.49**	0.21	1.00							
	pH_{24}	-0.20	-0.30	-0.06	0.25	0.31	0.25	0.22	0.23	0.02	-0.18	-0.20	-0.10	-0.07	1.00						
	WBSF	-0.05	0.01	0.12	-0.10	0.07	-0.04	-0.26	-0.12	-0.06	-0.09	0.07	0.06	-0.17	0.29	1.00					
	Cooking loss	0.04	0.04	-0.10	-0.16	0.14	0.06	-0.14	-0.10	0.30	-0.45*	0.32	0.26	0.14	0.24	0.23	1.00				
	Tenderness	0.08	0.18	-0.12	0.21	-0.16	-0.14	0.11	0.05	0.15	-0.08	0.31	0.08	0.33	-0.37*	-0.43*	0.25	1.00			
	Juiciness	0.28	0.12	0.26	0.31	-0.32	-0.33	0.31	0.28	0.02	0.23	0.02	-0.35	-0.23	-0.08	0.14	0.05	0.21	1.00		
	Flavour	0.33	0.28	0.20	0.30	-0.36	-0.40*	0.31	-0.11	-0.28	0.21	-0.31	-0.41*	-0.24	-0.01	0.14	0.03	0.05	0.66***	1.00	
	Acceptability	0.34	0.23	0.11	0.42*	-0.41*	-0.40*	0.24	0.21	0.10	0.12	0.17	-0.02	0.14	-0.28	-0.32	0.28	0.83***	0.52**	0.33	1.00

*$P < 0.05$, **$P < 0.01$, and *** $P < 0.001$.

2.4. Discussion

Producing pork with an increased IMF content is a major aim of the meat industry to meet consumer demand. In fact, the level of IMF influences meat quality, and the minimum value of 2.5% IMF has been proposed as necessary for sensory acceptability of pork (DeVol *et al*., 1988; Fernandez *et al*., 1999b). The increased IMF obtained in the present study (about 50%) for lean (crossbred) pigs fed RPD, not adjusted for lysine (17.5 *vs.* 13.1% crude protein), is in agreement with several previous studies (*e.g*., Da Costa *et al*., 2004; Wood *et al.*, 2004; Doran *et al.*, 2006). It was reported that under certain limits, the lower the dietary protein the greater the marbling and IMF content (Castell *et al.*, 1994; Goerl *et al*., 1995). However, in those earlier reports, it is not clear if the muscle lipogenic response was due to dietary protein reduction per se, reduction of dietary lysine levels, or both. In our experiment, the only dietary treatment that increased IMF in *longissimus lumborum* muscle was RPD in crossbred pigs. Thus, our results clearly indicate that it was the reduction of lysine availability in the diet that promoted IMF deposition in lean pig genotype.

In contrast to the crossbred genotype, neither dietary crude protein nor lysine reductions influenced IMF in Alentejano genotype, which was about twofold greater in the Alentejano than crossbred pigs. Lysine requirements of growing pigs are directly proportional to protein accretion rate (NRC, 1998). Studies in male Iberian pigs (Barea *et al.*, 2006), with phenotypic and genotypic similarities to Alentejano genotype (Pugliese & Sirtori, 2012), indicated that the reduced-lysine level used in the present study could be enough to meet the requirements for Alentejano genotype, which could explain the lack of effect on IMF in Alentejano males fed RPD. In addition, it is possible that breed-specific expression of muscle fiber types and key lipogenic enzymes occurs, which have been described as the most important factors influencing IMF in pigs (Guo *et al*., 2011).

The Alentejano and crossbred pigs growth rates were similar throughout the experiment (from 60 to 90 kg), although Alentejano breed had much greater body fat deposition, as indicated by greater IMF and shoulder, P_2, L6, and S2 backfat thickness values, and, consequently, less muscle mass, as indicated by less loin weight. Thus, the energy value of body weight gain is greater in Alentejano than crossbred pigs, which explains the enhanced ADFI and reduced G:F observed in Alentejano pigs, compared with the crossbred genotype. The genotype has been reported in several studies to induce distinct carcass traits, including marbling, loin muscle area, and percentage of lean meat (McLaren, Buchanan & Johnson, 1987; Pugliese & Sirtori, 2012). The difference between genotypes was expected because Alentejano is a native, no improved pig genotype, with a greater propensity to deposit fat because of a genetic heritage distinct from high-performing pigs, such as Large White, Landrace, and Pietrain.

In the present study, RPDL had no effect on ADG, backfat thickness, and loin weight, in both Alentejano and crossbred pigs. Nonetheless, when the dietary crude protein level was reduced without correction for lysine content (RPD), ADG was affected in crossbred but not in Alentejano pigs. In general, low-protein (Teye *et al*., 2006) and lysine-deficient (O'Connell, Lynch & O'Doherty, 2006) diets decrease ADG in pigs during the lean growth phase. Regarding the effects of lysine reduction on growth performance and carcass traits, it is possible that most of the results published in the literature are not due to dietary protein reduction per se, but rather to the consequent deficiency in dietary Lys supply.

The appearance of pork at retail is mainly influenced by IMF and colour. Meat colour is a very important factor that influences the purchase decision of the consumer, as it is perceived as a measure of freshness and quality (Khliji, van de Ven, Lamb, Lanza & Hopkins, 2010). Furthermore, published data indicate that despite its poor eating quality, consumers prefer leaner pork (Brewer, Zhu & McKeith, 2001). In general, Alentejano meat has greater IMF, is darker (lower L*), and redder (greater a*) than meat obtained from improved commercial pig genotypes. This trend was confirmed in the present study and could help to distinguish Alentejano meat from other pork at retail. However, the preference of consumers for a deeper pink colour, rather than a pale white meat, could vary among countries and even within a country.

The important role of IMF in pork palatability has been described by several authors (*e.g*., DeVol *et al*., 1988; Hocquette *et al*., 2003; Lonergan *et al.*, 2007; Cannata *et al*., 2010; Hocquette *et al.*, 2010). The Alentejano genotype had greater IMF content than crossbred pigs, which was accompanied by greater sensory panel ratings. Although not statistically significant, the greater tenderness, juiciness, flavour, and acceptability scores within crossbred genotype were found when the level of IMF in meat increased about 1.3% because of the reduced dietary lysine level (RPD). However, this increment was apparently not enough to affect pork eating quality. Indeed, an increase in pork IMF content is not always related to a greater eating quality (Rincker, Killefer, Ellis, Brewer & McKeith, 2008) because other genetic factors, including the ultimate pH, can play an important role.

It is worth noting that the pH_{24} increase was associated with lower tenderness scores in crossbred but not in Alentejano pigs, which could be related to differences in IMF content. In fact, the relationships between meat lipid content and sensory traits are ultimately pH dependent (Lonergan *et al*., 2007). However, in contrast to previous studies where pH had noticeable effects on sensory traits (Zhang *et al.*, 2007; Cannata *et al.*, 2010) and cooking loss (DeVol *et al*., 1988), excluding tenderness in crossbred pigs, variations in pH_{24} had no influence on pork quality in the present work.

A positive eating experience for pork meat depends on several attributes. The most pertinent sensory factors are tenderness, flavour, and juiciness. Tenderness has been identified as the

most important attribute to determine pork acceptability (Resurreccion, 2004; Fortin, Robertson & Tong, 2005; Cannata *et al*., 2010). In the present study, pork acceptability was mainly defined by tenderness and juiciness with flavour presenting a minor role in both genotypes. The WBSF is considered a very reliable indicator of tenderness (Skelley, Handlin & Bonnette, 1973). However, this has not been fully confirmed in this study because the correlation between meat WBSF and tenderness was lower than 0.5, and only statistically significant in crossbred pig genotype.

The IMF from Alentejano pigs had a greater MUFA to SFA ratio than that from crossbred pigs, which is consistent with previous reports, indicating that native genotypes (Pugliese & Sirtori, 2012), such as Alentejano breed, show a great predisposition to deposit MUFA, mainly 18:1*c*9, whereas high-performing pigs display greater quantities of SFA. In general, eating quality traits improve as IMF content and MUFA percentage increase, and PUFA proportion decreases (Cameron & Enser, 1991). This trend is supported by these observed correlations: the 18:1*c*9 was positively associated, whereas 18:2*n-6* and 20:4*n-6* were negatively correlated, with flavour, acceptability or both. Increased PUFA have often been reported to result in off-flavours, particularly after reheating pork. This is because PUFA has a greater susceptibility to produce undesired volatile compounds during cooking (Larick, Turner, Schoenherr, Coffey & Pilkington, 1992). In addition, Rhee, Davidson, Cross & Ziprin (1990) reported increased taste panel scores for tenderness and juiciness attributes, which parallel with a greater concentration of 18:1 in muscle lipids. In contrast, no statistically significant correlations between fatty acid composition and tenderness or juiciness were detected in Alentejano and crossbred pig genotypes. This is in line with other findings where an increase of 18:1 had no influence on pork eating quality (St. John *et al*., 1987).

A weak positive correlation between IMF content and flavour was previously reported (Channon, Kerr & Walker, 2004; Fortin *et al*., 2005). The same relationship was observed in both genotypes, but the correlation only reached statistical significance in Alentejano pigs. Despite the contribution of fatty acid composition to pork flavour observed in both genotypes, its influence on meat acceptability was only observed in crossbred pigs. These results indicate that the contribution of fatty acid profile to pork acceptability is far from linear and could depend on IMF content. However, the influence of IMF to sensory quality is regarded as contradictory. Some studies indicated that IMF has a minor contribution to sensory characteristics (Wood *et al*., 1996; van Laack *et al*., 2001), whereas others found that lipid content has an important role in eating experience (Brewer *et al*., 2001; Wood *et al*., 2004; Fortin *et al*., 2005; Hocquette *et al*., 2010). The IMF content was not correlated with pork acceptability for both pig genotypes. It is possible that a threshold value for IMF exists, rather than a simple, linear relationship between IMF and tenderness (DeVol *et al.*, 1988).

2.5. Conclusions

In conclusion, results of this study indicate that the use of reduced crude protein diets during the growing-finishing phase of pigs is a potential approach for increasing IMF content in pork. In addition, the data strongly indicate that the increased IMF deposition is due to lysine restriction in pig diets. However, the response to this dietary crude protein/lysine reduction seems to depend ultimately on the genetic background of the pig (*i.e.*, it was only effective in lean pig genotypes). Moreover, the small increment in pork sensory traits obtained with the increased IMF content indicates that this dietary approach may have limited use for meat industry and consumers, particularly in pig genotypes with a relatively high marbling level.

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Chapter 3

DIFFERENTIAL EFFECTS OF REDUCED PROTEIN DIETS ON FATTY ACID COMPOSITION AND GENE EXPRESSION IN MUSCLE AND SUBCUTANEOUS ADIPOSE TISSUE OF ALENTEJANA PUREBRED AND LARGE WHITE × LANDRACE × PIETRAIN CROSSBRED PIGS

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Contribution of Marta S. Madeira to this article:
Marta S. Madeira collaborated in the animal experiment, participated in the tissue sampling, did the IMF and fatty acids analysis, data processing and statistical analysis of fatty acids results. In addition, Marta S. Madeira collaborated in the interpretation of the results and in the writing of the manuscript.

*These co-authors contributed equally for this paper.

Differential effects of reduced protein diets on fatty acid composition and gene expression in muscle and subcutaneous adipose tissue of Alentejana purebred and Large White × Landrace × Pietrain crossbred pigs

Abstract

The present study assessed the effect of pig genotype (fatty *vs.* lean) and dietary protein and lysine levels (normal *vs.* reduced) on IMF content, SAT deposition, fatty acid composition and mRNA levels of genes controlling lipid metabolism. The experiment was conducted on sixty intact male pigs (thirty Alentejana purebred and thirty Large White × Landrace × Pietrain crossbred), from 60 to 93 kg of live weight. Animals were divided into three groups fed with the following diets: control diet equilibrated for lysine (17.5% crude protein and 0.7% lysine), RPD equilibrated for lysine (13.2% crude protein and 0.6% lysine) and RPD not equilibrated for lysine (13.1% crude protein and 0.4% lysine). It was shown that the RPD increased fat deposition in *longissimus lumborum* muscle in the lean but not in the fatty pig genotype. It is strongly suggested that the effect of RPD on the *longissimus lumborum* muscle of crossbred pigs is mediated via lysine restriction. The increase in IMF content under the RPD was accompanied by increased *SCD* and *PPARγ* mRNA levels. RPD did not alter backfat thickness, but increased the total fatty acid content in both lean and fatty pig genotype. The higher amount of SAT in fatty pigs, when compared with the lean ones, was associated with the higher expression levels of *ACACA*, *CEBPα*, *FASN* and *SCD* genes. Taken together, the data indicate that the mechanisms regulating fat deposition in pigs are genotype and tissue specific, and are associated with the expression regulation of the key lipogenic genes.

Keywords: pigs, reduced protein diet, intramuscular fat, fatty acid composition, lipid metabolism

3.1. Introduction

Pork is one of the most consumed meats in the European Union, with 22 010 778 tons of carcass produced in 2010 (Eurostat, 2012). However, as the consequence of genetic selection towards reduced subcutaneous fat, particularly in the case of white European breeds (Large White and Landrace), the amount of intramuscular or marbling fat (IMF) in commercial crossbred pigs has also been dramatically reduced (Jeremiah *et al*., 1999). Conversely, some pig breeds, like Alentejana and Iberian, have typically large amounts of subcutaneous and IMF, which are very precociously deposited in the carcasses (Daza, Mateos, Rey, Ovejero & López-Bote, 2007). IMF is one of the key meat quality traits. The sensory properties of pork, such as juiciness, tenderness and overall acceptability, are negatively affected when IMF is reduced below 2% (Eikelenboom & Hoving-Bolink, 1994a). It was proposed that acceptable pork eating quality requires a minimum IMF of 2.5% (De Vol *et al.*, 1988). However, according to Daszkiewicz, Bak & Denaburski (2005), about 84% of the carcasses from commercial pig genotypes have a *longissimus lumborum* muscle fat content below the level required for acceptable eating quality. In contrast to beef, IMF in pork is usually not visible and, hence, an increase in IMF should not result in the rejection of the meat by consumers due to marbling (Mourot & Hermier, 2001). In addition, it is well known that fatty acid composition of IMF plays an important role in meat quality, and therefore an appropriate proportion of SFA, MUFA and PUFA should be maintained in order to assure superior eating quality and nutritional value (Wood *et al.*, 2008). Therefore, production of pork with high amounts of IMF and a balanced fatty acid composition, without an increase in subcutaneous fat (improved fat partitioning), is highly desirable for the pig industry and consumers.

In pigs, the use of RPDs (Doran *et al.*, 2006) or low lysine levels (D'Souza *et al.*, 2008), has been proved to be the most successful nutritional strategy to enhance fat accumulation in muscle without a significant effect on SAT. Although the principle of these strategies is to restrict muscle development, the mechanisms involved in the increasing of IMF content remain unknown (Hocquette *et al.*, 2010). One of the possible explanations might be the tissue-specific stimulation of expression of lipogenic enzymes under RPD, which, in turn, could lead to the increase of *de novo* fatty acid synthesis. One of the key lipogenic enzymes is SCD, which catalyses the rate-limiting step of MUFA biosynthesis. Da Costa *et al.* (2004) showed that a RPD with a low lysine level increased SCD transcriptional rate in pig muscles. In line with this, Doran *et al.* (2006) demonstrated that this increase in the transcriptional rate is followed by an increase in SCD protein expression and activity in muscles, but not in SAT from a commercial lean pig genotype (Duroc × Large White × Landrace). However, it remains unknown whether a combined reduction of dietary protein and lysine levels is

required to increase IMF, and whether responses of fatty pig genotypes to RPD are similar to those of lean pig genotypes.

In addition to SCD, there are a number of other key enzymes and transcription factors involved in lipid metabolism. These factors determine the rates of *de novo* fatty acid biosynthesis, fat uptake from blood, transport of fatty acids in adipocytes and lipid degradation. ACACA (Liu *et al.*, 1994) and FASN (Clarke, 1993) are the key lipogenic enzymes controlling the rates of SFA biosynthesis. LPL is the rate-limiting enzyme for the conversion of chylomicrons and VLDL into chylomicron remnants and LDL in tissues. Therefore, LPL controls triacylglycerols (TAG) partitioning between adipose tissue and muscle, thereby increasing fattening or providing energy in the form of fatty acids for muscle growth (Hocquette *et al.*, 1998). Furthermore, FABP4 is responsible for fatty acid transport in adipocytes (Hocquette *et al.* 2010). Moreover, CRAT is the rate-limiting enzyme of lipid catabolism, transporting fatty acid esters from cytosol to mitochondria for β-oxidation (Van der Leij *et al.*, 2000), whereas PPARα is a major inducer of fatty acid oxidation (Poulsen *et al.*, 2012). It is also known that the transcription factors, SREBP1, CEBPα and PPARγ, are involved in the control of lipid metabolism in adipose tissue via regulation of expression of key enzymes and proteins controlling adipogenesis and lipogenesis (Kokta *et al.*, 2004; Hocquette *et al.*, 2007; Zhao *et al.*, 2010). The effects of dietary protein and lysine levels on the expression of genes encoding for lipid-metabolising enzymes are largely unknown.

To summarise, the genotype- and tissue-specific effects of RPD on fat partitioning and fatty acid composition in pigs, the interaction between dietary protein and lysine levels and the role of lipogenic enzymes and nuclear transcription factors in regulation of these effects remain to be elucidated. Therefore, in the present chapter, we tested the following hypothesis: (1) the effect of RPD on fat partitioning between the muscle and subcutaneous depots is genotype specific; (2) the effect of RPD on fat partitioning is realised via the restriction in dietary lysine level; (3) the tissue-specific effect of RPD is mediated via the expression of key genes controlling lipid metabolism. To answer to the earlier questions, two distinct pig genotypes were chosen for the present study, the fatty Alentejana purebred and a lean commercial crossbred.

3.2. Material and Methods

3.2.1. Animals and diets

The present experiment was conducted at the facilities of UIPA-INIAV, and all the experimental procedures involving animals were reviewed by the Ethics Commission of the CIISA/FMV and approved by the Animal Care Committee of the National Veterinary Authority

(Direcção-Geral de Veterinária) following the appropriate European Union guidelines (Directive 86/609/EEC). A total of thirty Alentejana purebred and thirty commercial crossbred (50% Large White, 25% Landrace and 25% Pietrain) entire male pigs with an average initial body weight of 59.9 ± 1.97 kg (mean ± standard deviation) were used. Animals were fed a standard concentrate diet from weaning until the beginning of the experiment. Thereafter, animals from each genotype were randomly assigned to one of the three diets in a 2 × 3 factorial arrangement (two genotypes and three diets). The experimental diets were isoenergetically formulated (13.5 MJ ME/kg calculated according to the NRC (1998)) and differed in crude protein and lysine contents, as follows: 17.5% of crude protein and 0.7% of lysine (control diet); 13.2% of crude protein and 0.6% of lysine (reduced protein diet equilibrated for lysine, RPDL); and 13.1% of crude protein and 0.4% of lysine (reduced protein diet not equilibrated for lysine, RPD). L-Lysine was added to the RPDL diet to equilibrate the level of this amino acid with the control diet. The ingredients, chemical composition and fatty acid profile of the experimental diets are shown in Table 3.1. The animals were housed in two pens of four pigs each and one pen of two pigs per treatment (n=10). During the experiment, the animals were fed individually twice a day and had access to water *ad libitum*. Feed offered and refusals were recorded daily in order to calculate feed intake. Pigs were weighed weekly, just before feeding, throughout the experiment.

3.2.2. Slaughter and sampling

Feed was removed 17-19 h before the slaughter of the animals. Pigs were slaughtered at an average live body weight of 93.4 ± 2.42 kg, with no significant differences (P>0.05) among animal groups, at the UIPA Experimental Abattoir (INIAV). Immediately after electrical stunning and exsanguination, samples of the *longissimus lumborum* muscle and SAT for gene expression analysis were collected from the right side of the carcass at the first lumbar vertebra level, rinsed with sterile RNAse-free cold saline solution, cut into small pieces (thickness of about 0.3 cm), stabilised in RNA Later solution (Qiagen, Hilden, Germany) and stored at -80 ºC until analysis. For the determination of IMF and fatty acid composition, *longissimus lumborum* muscle and SAT samples were collected after slaughter from the right side of the carcass between the third and fifth lumbar vertebras. Muscle was collected and trimmed of visible connective and adipose tissues before blending in a food processor. The samples of muscle and SAT were vacuum packed and stored at -20 ºC until analysis. Backfat thickness was measured in the left carcass side at shoulder, P_2 (last rib position), last lumbar vertebra and second sacral vertebra locations.

Table 3.1 - Ingredients, chemical and fatty acid compositions of the experimental diets.

	Control	RPDL	RPD
Ingredients (%)			
Barley	40.0	50.0	50.0
Wheat	26.1	29.5	29.0
Soybean meal	24.8	11.7	11.8
Corn	5.0	5.0	5.0
Soybean oil	1.6	1.5	1.5
Calcium carbonate	1.1	1.1	1.1
Vitamin-trace mineral premix[1]	0.4	0.4	0.4
Salt	0.4	0.4	0.4
Dicalcium phosphate	0.2	0.4	0.4
L-Lys	0.0	0.2	0.0
Phytase mixture[2]	0.1	0.1	0.1
Acid mixture[3]	0.1	0.1	0.1
Fermentation products[4]	0.1	0.1	0.1
Mold inhibitor mixture[5]	0.05	0.05	0.05
Antioxidant mixture[6]	0.003	0.003	0.003
Chemical composition (% diet)			
Dry matter	89.1	88.9	89.0
Crude protein	17.5	13.2	13.1
Starch	47.2	54.9	55.2
Crude fat	3.1	2.9	2.9
Crude fibre	4.9	4.1	4.0
Ash	4.4	3.9	4.0
L-Lysine	0.7	0.6	0.4
Calcium	0.82	0.78	0.84
Phosphorus	0.37	0.37	0.37
Metabolisable energy (MJ ME/kg)	13.3	13.6	13.5
Lysine/ME	0.049	0.041	0.030
Fatty acid composition (% total fatty acids)			
14:0	0.1	0.1	0.2
16:0	17.3	18.7	19.6
16:1*c*9	0.2	0.2	0.2
18:0	2.6	2.6	2.6
18:1*c*9	19.0	18.9	19.3
18:1*c*11	1.5	1.5	1.6
18:2*n-6*	52.5	51.4	50.2
18:3*n-3*	4.9	4.6	4.3
20:0	0.3	0.3	0.3
20:1*c*11	0.4	0.5	0.5

Dietary treatments: Control, normal protein diet equilibrated for lysine; RPDL, reduced protein diet equilibrated for lysine level; RPD, reduced protein diet not equilibrated for lysine level.

[1]Vita Tec (Tecadi, Santarém, Portugal). Provided per kilogram of diet: vitamin A, 6,000 IU; vitamin D3, 1,500 IU; vitamin E (acetate dl-alpha-tocopherol), 15 mg; vitamin B2, 0.3 mg; vitamin B12, 3.75 mg; biotin, 0.1 mg; calcium pantothenate, 12 mg; nicotinic acid, 15 mg; folic acid, 0.75 mg; choline chloride, 200 mg; Cu (cupric sulfate pentahydrate), 15 mg; Zn (zinc oxide), 100 mg; Mn (manganese oxide), 35 mg; I (potassium iodide), 0.7 mg; Co (basic cobaltous carbonate mono hydrous), 0.05 mg; Se (sodium selenite), 0.2 mg; Fe (ferrous carbonate), 80 mg; and BHT, 0.2 mg.

[2]3-phytase and calcium carbonate (Tecaphos 500 g; Tecadi).

[3]Formic acid, propionic acid, citric acid, and calcium salts (Ultracid V Dry EU; Tecadi).

[4]Fermentation product of *Aspergillus niger* fungus using wheat middlings as substrate combined calcium carbonate (Graintec TS; Tecadi).

[5]Hydrated aluminum silicates, Na, yeast extracts, calcium propionate, calcium formate, and antioxidant (Unike Plus Dry; Tecadi).

[6]Ethoxyquin, propyl gallate, and citric acid (Oxi-Nil Dry Premix; Tecadi).

3.2.3. Feed analysis

Feed samples, collected four times during the experiment (in the beginning and on a 3 week regular period), were analysed for dry matter by drying a sample at 100 ºC to a constant weight. Nitrogen content was determined by Kjeldahl (AOAC, 2000) and crude protein was calculated as 6.25 × N. Crude fibre was determined by the procedure described by the AOAC (2000). The samples were extracted with petroleum ether, using an automatic Soxhlet extractor (Gerhardt Analytical Systems, Königswinter, Germany), to determine crude fat. Determination of ash and starch contents was carried out according to the procedures described by the AOAC (2000) and Clegg *et al.* (1956), respectively. Gross energy in the feed was determined by adiabatic bomb calorimetry (Parr 1261, Parr Instrument Company, Moline, IL, USA). Fatty acid methyl esters (FAME) of feed samples were analysed by one-step extraction and transesterification, using heptadecaenoic acid (17:0) as an internal standard (Sukhija & Palmquist, 1988). Total amino acids were extracted from feed according to the method described by the AOAC (2000). The extract was analysed by HPLC (Agilent 1100, Agilent Technologies, Avondale, PA, USA) to quantify amino acids in the feed, including lysine, according to the procedure reported by Henderson *et al.* (2000).

3.2.4. Intramuscular fat and fatty acid composition

The *longissimus lumborum* muscle and SAT samples were lyophilised (-60 °C and 2.0 hPa) to constant weight using a lyophilisator (Edwards High Vacuum International, West Sussex, UK), kept dry at -20 °C and analysed within two weeks. The total fat content of muscle samples (IMF) was determined using fresh samples by hydrolysis with 4 M HCl followed by Soxhlet extraction during 6 h with petroleum ether (AOAC, 2000). For fatty acid analysis of *longissimus lumborum* muscle and SAT samples, FAME were extracted from the lyophilised samples (approximately 250 and 50 mg, respectively), according to the Folch & Stanley (1957) method, using dichloromethane and methanol (2:1, v/v) instead of chloroform and methanol (2:1, v/v), as described by Carlson (1985). All the extraction solvents contained 0.01% butylated hydroxytoluene as an antioxidant. Fatty acids were converted to methyl esters by a combined transesterification procedure with NaOH in anhydrous methanol (0.5M), followed by HCl:methanol (1:1, v/v), at 50 °C for 30 and 10 min, respectively, according to Raes *et al.* (2001). Quantification of FAME in muscle and SAT was performed using a gas chromatograph HP6890A (Hewlett-Packard), equipped with a flame ionisation detector (GC-FID) and a CP-Sil 88 capillary column (100 m × 0.25 mm inner diameter, 0.20 µm film thickness; Chrompack, Varian Inc., Walnut Creek, CA, USA), using the conditions described in Alves & Bessa (2009). The quantification of total FAME was done using

nonadecanoic acid (19:0) as the internal standard. Results for each fatty acid were expressed as a percentage of the sum of detected fatty acids (% total fatty acids).

3.2.5. RNA isolation and complementary DNA synthesis

Total RNA was isolated and purified from muscle and SAT using the Qiagen RNeasy fibrous tissue mini kit (Qiagen) and Qiagen RNeasy lipid tissue mini kit (Qiagen), respectively. Prior to RT-PCR, the total RNA samples were treated with DNAse I (Qiagen). All the procedures were performed in accordance with the manufacturer's protocols. RNA was quantified using a NanoDrop ND-2000c spectrophotometer (Nanodrop, Thermo Fisher Scientific, Willmington, DE, USA). The A260/280 ratios were between 1.9 and 2.1. Ethidium bromide staining of 18S and 28S ribosomal bands was used to verify the sample integrity. Reverse transcription was performed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Briefly, each 20 µl RT reaction containing 1 µg of DNase-treated total RNA template, 50 nM random RT primer, 1×RT buffer, 0.25 mM of each deoxyribonucleotide triphosphate (dNTP), 3.33 U/µl multiscribe reverse transcriptase (RT) and 0.25 U/µl RNase inhibitor, was submitted to 25 ºC for 10 min, 37 ºC for 120 min and 85 ºC for 5 min. The complementary DNA solution obtained was divided in aliquots and stored at -20 ºC until further analysis.

3.2.6. Real-time quantitative PCR

Gene-specific intron-spanning primers were designed using Primer3 (http://frodo/wi.mit.edu/primer3) and Primer Express Software v. 2.0 (Applied Biosystems) based on *Sus scrofa* sequences (http://www.ncbi.nlm.nih.gov). Primers were synthesized commercially by NZYTech (Lisbon, Portugal). Sequence homology searches against the database of GenBank showed that these primers matched only with the sequence to which they were designed. To ensure optimal DNA polymerisation efficiency, the amplicon length ranged between 71 and 138 bp. Before performing the real-time quantitative PCR experiments, a conventional PCR was carried out for all genes investigated in order to test the primers and verify the amplified products. To confirm the identity of amplified fragments, PCR products were sequenced and homology searches were performed with Blast (http://www.ncbi.nlm.nih.gov/blast). In order to find the most stable endogenous control in SAT and *longissimus lumborum* muscle, five commonly used housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 60S ribosomal protein L27 (*RPL27*), ornithine decarboxylase antizyme 1 (*OAZ1*), ribosomal protein large P0 (*RPLP0*) and 40S ribosomal protein S29 (*RPS29*) were used to normalise the results of target genes.

Expression level stability of housekeeping genes was analysed using the geNorm (http://medgen.ugent.be/~jrdesomp/genorm) (Vandesompele *et al.*, 2002) and NormFinder (http://www.mdl.dk/publicationsnormfinder.htm) (Andersen, Jensen & Orntoft, 2004) software packages as described in their manuals. The *RPLP0* and *RPS29* genes were selected as the most stable pair of internal controls for normalisation. The sequence of primers (including annealing temperatures), GenBank accession numbers, PCR efficiency, regression coefficient and span exons for PCR products are provided in Table 3.2. PCR efficiency was calculated for each amplicon using StepOnePlus PCR System software (Applied Biosystems), by amplifying 5-fold serial dilutions of pooled complementary DNA and run in triplicate. All primer sets exhibited an efficiency that ranged between 90 and 110%, and correlation coefficients were higher than 0.99. Real time quantitative PCR were carried out using MicroAmp Optical ninety-six-well plates (Applied Biosystems) in a StepOnePlus thermocycler (Applied Biosystems) under standard cycling conditions. The 12.5 µl PCR mixtures contained 6.25 µl of 2×Power SYBR Green PCR Master Mix (Applied Biosystems), 160 nM of forward and reverse primers and 2 µl of diluted complementary DNA as template. No transcription and no template samples were used as controls. The primer specificity and the formation of primer-dimers were confirmed by melt curve analysis and agarose gel electrophoresis. All analyses were performed in duplicate, and the relative amounts for each target gene was calculated using the geometric mean of *RPLP0* and *RPS29* as a normaliser. The relative expression levels were calculated as a variation of the Livak & Schmittgen (2001) method, corrected for variation in amplification efficiency, as described by Fleige *et al.* (2006).

3.2.7. Statistical analysis

For IMF content and fatty acid composition, all experimental groups were considered. As the RPDL had no significant effect on IMF and SAT deposition, relative to the control diet, gene expression analysis was performed only on four experimental groups (Alentejano and crossbred pigs fed with the control and RPD diets). All data were checked for normal distribution and variance homogeneity. As variance heterogeneity was detected for most of the variables, data were analysed using Proc MIXED of the SAS software package (SAS, 2009) (version 9.2; SAS Institute), with a model including the genotype, diet and their respective interaction as fixed effects and the repeated statement considering the group option to accommodate the variance heterogeneity. The level of significance was set at $P<0.05$.

Table 3.2 - Characterisation of the selected genes used in the real-time quantitative PCR assay.

Gene symbol	Full gene name	GenBank accession number	PCR efficiency SAT/LL[1] muscle	Regression coefficient (r^2) SAT/LL muscle	Forward primer	Reverse primer	Product size (bp)	Spanned coding exons	Annealing tempera-ture (ºC)
Key lipid metabolism genes									
ACACA	Acetyl-CoA carboxylase, alpha	NM_001114269	97.1/90.5	0.997/0.996	ggccatcaaggacttcaacc	acgatgtaagcgccgaactt	120	46-47	58.0/58.5
CEBPA	CCAAT/enhancer binding protein (C/EBP) alpha	XM_003127015	91.78/100.0	0.994/0.990	ggccagcacacacacattaga	cccccaaagaagagaaccaag	71	1	58.2/58.7
CRAT	Carnitine O-acetyltransferase	NM_001113047	98.86/92.96	0.996/0.999	ggcccaccgagcctacac	atggcgatggcgtaggag	138	12-13	60.0/58.2
FABP4	Fatty acid binding protein 4	NM_001002817	98.0/91.77	1.0/0.996	gggccaggaatttgatgaag	ctttccatcccacttctgcac	103	2-3	58.0/58.2
FASN	Fatty acid synthase	NM_001099930	92.45/91.07	0.999/0.999	acaccttcgtgctggcctac	atgtcggtgaactgctgcac	112	40-41	58.8/58.3
LPL	Lipoprotein lipase	NM_214286	93.15/91.15	0.999/0.997	atctgcgggatacaccaagc	ccaaggctgtatcccaggag	110	3-4	58.6/58.2
PPARα	Peroxisome proliferator-activated receptor alpha	NM_001044526	97.92/100.16	0.995/0.998	tttccctctttgtggctgct	ggggtggttggtctgcaag	128	5-6	58.5/59.9
PPARγ	Peroxisome proliferator-activated receptor gamma	NM_214379	97.88/98.5	0.999/0.993	gagggcgatcttgacaggaa	gccacctctttgctctgctc	124	6-7	58.8/58.8
SCD	Stearoyl-CoA desaturase	NM_213781	94.86/93.16	0.997/0.998	agccgagaagctggtgatgt	gaagaaaggtggcgacgaac	140	5-6	58.5/58.4
SREBP1	Sterol regulatory element binding protein 1	NM_214157	102.0/96.43	0.999/0.999	gtgctggcggaggtctatgt	aggaagaagcgggtcagaaag	96	11-12	58.8/58.6
Housekeeping genes									
RPLP0	Ribosomal phosphoprotein large, P0 subunit	NM_001098598	95.36/97.14	0.998/0.999	tccaggctttaggcatcacc	ggctcccactttgtctccag	95	4-5	58.7/58.3
RSP29	Ribosomal protein S29	NM_001001633	96.65/97.57	1.0/1.0	ggtcagggttctcgctcttg	cactggcggcacatattgag	120	1-2	58.5/58.7

[1] SAT, subcutaneous adipose tissue; LL, *longissimus lumborum*.

The need for covariate adjustment was explored using age, live and slaughter weights, IMF and P_2 as covariates, but only IMF and P_2 revealed to be significant for several variables. Thus, IMF and P_2 were retained as covariates for some muscle and SAT variables, respectively. For each variable, where the use of a covariate was justified, the structure of the covariate model was determined according to the procedures described by Milliken & Johnson (2002) and ranged from a simple slope model to individual slopes for each diet × genotype combinations. The adjusted variables and their covariance models are identified in the footnotes of the tables. As large differences in covariate ranges were intrinsically associated to each genotype, the variable was adjusted and compared with the mean covariate value of each genotype (Milliken & Johnson, 2002). When significant effects were detected, least square means (LSMEANS) were determined using the LSMEANS option and compared using the probability difference procedure adjusted for multiple comparisons using the Tukey-Kramer method.

Pearson correlation matrices were computed using the PROC CORR of SAS. When needed, adjusted variables to the common mean IMF in muscle and the common mean P_2 in SAT were used to compute Pearson correlations.

3.3. Results

The results of this experiment regarding pigs' performance, carcass traits and sensory quality of meat were obtained (Madeira *et al.*, 2013a, Chapter 2). However, here we present and discuss the effects of RPD, with (RPDL) or without (RPD) equilibrated levels of lysine, on fatty acid content and composition of muscle and SAT from lean (commercial crossbred) and fatty (Alentejana purebred) pig genotypes. Furthermore, in order to elucidate the mechanisms underlying fat deposition in *longissimus lumborum* muscle and SAT obtained for the RPD in crossbred pigs, the expression level of genes encoding for key lipogenic enzymes and transcription factors involved in lipid metabolism was also assessed. As no significant effects (P>0.05) in IMF deposition were obtained for the RPDL, when compared with the control diet, the expression level of key genes involved in lipid metabolism were not investigated for the experimental groups fed this diet.

3.3.1. Intramuscular fat and fatty acid composition of muscle

Results of IMF, fatty acid composition, partial sums of fatty acids and related ratios in the *longissimus lumborum* muscle are presented in Table 3.3. In relation to IMF content, a significant interaction between genotype and diet (P=0.037) was observed, with no dietary effect for Alentejano pigs but with an increase of IMF by 40% for the RPD in crossbred animals. In contrast, the RPDL did not increase (P>0.05) IMF, neither in Alentejano nor in crossbred pigs.

In all experimental groups, the predominant fatty acids in IMF were 18:1*c*9 (33-38% of total FAME), 16:0 (23-26%), 18:0 (12-14%), 18:2*n-6* (7-12%) and 18:1*c*11 (5-6%). It should be noted that 18:1 *trans* represents the sum of 18:1 *trans* 6 to *trans* 11. The term "others" refers to unidentified minor fatty acids and the dimethylacetals 16:0, 18:0 and 18:1, which are derived from plasmalogens. The genotype and diet interaction influenced only three fatty acids (12:0, 16:1*c*9 and 18:1*c*11). The genotype affected fourteen of the nineteen fatty acids identified. The proportion of 16:0 (P<0.001), 18:0 (P<0.001), 18:1*c*9 (P<0.001) and 20:0 (P=0.003) was highest in Alentejana purebred animals, when compared with the crossbred genotype. This is in contrast to the 14:0, 17:0, 18:2*n-6*, 18:3*n-3*, 20:3*n-6* and 20:4*n-6* fatty acids, which were highest in crossbred pigs. In addition, the dietary protein and lysine levels affected eight individual fatty acids in the *longissimus lumborum* muscle. The proportion of 16:0 was higher (P=0.001) in the pigs fed RPD, when compared with the animals fed control diet. Contrarily, 16:1*c*7 (P=0.026), 18:2*n-6* (P=0.010), 18:3*n-3* (P=0.004), 20:2*n-6* (P=0.015) and 20:3*n-6* (P=0.002) were lower in the RPD than in the control diet.

Regarding partial sums of fatty acids (Table 3.3), the observed patterns reflect the values described earlier for the major individual fatty acids of each group. Both, the genotype (P<0.001) and the diet influenced SFA (P≤0.001), PUFA (P=0.009) and *n-6* PUFA (P=0.010). The proportion of SFA was higher in the RPD relative to the control diet, while the proportions of PUFA and *n-6* PUFA and the PUFA/SFA ratio were lower in the RPD.

3.3.2. Fatty acid content and composition of subcutaneous adipose tissue

Table 3.4 shows backfat thickness at P_2 site, total fatty acids, fatty acid composition and related indices for SAT. Regarding P_2 backfat thickness, which is the most representative location (Teye *et al.*, 2006), a significant effect of genotype (P<0.001) was observed, in contrast to the diet (P=0.318), with values for Alentejano pigs being 90% higher than those obtained for crossbred animals. Similar values for backfat thickness in relation to dietary treatment were obtained at shoulder, last lumbar vertebra and second sacral vertebra locations (Madeira *et al*., 2013a, chapter 2). Regarding the total fatty acids (expressed as

Table 3.3 - Effect of the reduced protein diets equilibrated (RPDL) and not equilibrated (RPD) for lysine levels on intramuscular fat (IMF; % muscle), fatty acid composition (% total fatty acids), partial sums of fatty acids and related ratios in the *longissimus lumborum* muscle of Alentejano and crossbred pig genotypes.

	Alentejano						Crossbred						Significance level		
	Control		RPDL		RPD		Control		RPDL		RPD		Genotype	Diet	Genotype × diet
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE			
IMF	4.16^{b}	0.360	5.79^{b}	0.916	4.47^{b}	0.387	2.68^{a}	0.280	2.16^{a}	0.158	3.74^{b}	0.346	<0.001	0.143	0.037
Fatty acid composition															
12:0	0.09^{ab}	0.009	0.07^{a}	0.003	0.08^{ab}	0.005	0.08^{ab}	0.005	0.09^{b}	0.002	0.08^{a}	0.004	0.483	0.602	0.004
14:0*	1.31	0.047	1.40	0.043	1.45	0.042	1.52	0.041	1.51	0.072	1.46	0.053	0.011	0.661	0.125
16:0*	25.3	0.35	26.0	0.11	26.1	0.19	23.6	0.12	25.1	0.49	24.1	0.35	<0.001	0.001[1]	0.305
16:1*c*7*	0.25	0.011	0.22	0.010	0.21	0.009	0.24	0.009	0.24	0.016	0.21	0.012	0.621	0.026[2]	0.518
16:1*c*9*	2.76^{a}	0.102	3.06^{ab}	0.093	3.14^{ab}	0.091	3.20^{b}	0.089	3.43^{b}	0.156	2.93^{ab}	0.115	0.031	0.060	0.004
17:0†	0.23	0.017	0.19	0.011	0.18	0.008	0.29	0.023	0.28	0.023	0.31	0.038	<0.001	0.286	0.416
18:0	13.3	0.24	13.5	0.13	13.3	0.16	11.7	0.19	12.3	0.25	12.5	0.27	<0.001	0.091	0.213
18:1*t*	0.13	0.009	0.15	0.007	0.15	0.006	0.16	0.012	0.15	0.009	0.15	0.004	0.236	0.895	0.421
18:1*c*9†	37.5	0.64	37.6	0.66	37.7	0.63	34.4	0.63	34.0	0.64	35.8	0.65	<0.001	0.307	0.457
18:1*c*11*	5.21^{a}	0.242	5.80^{ab}	0.221	5.79^{ab}	0.217	6.37^{b}	0.213	5.69^{ab}	0.372	5.72^{ab}	0.273	0.127	0.980	0.019
18:2*n-6**	8.04	0.447	7.00	0.408	6.94	0.400	10.6	0.39	9.07	0.686	9.37	0.404	<0.001	0.010[3]	0.857
18:3*n-3**	0.37	0.018	0.34	0.016	0.33	0.016	0.44	0.016	0.38	0.028	0.37	0.020	0.003	0.004[2]	0.480
20:0	0.17	0.008	0.16	0.007	0.16	0.006	0.14	0.013	0.13	0.006	0.14	0.007	0.003	0.577	0.804
20:1*c*11†	0.66	0.027	0.65	0.027	0.64	0.027	0.65	0.026	0.57	0.027	0.63	0.027	0.126	0.289	0.332
20:2*n-6**	0.24	0.013	0.21	0.013	0.20	0.013	0.33	0.013	0.31	0.014	0.29	0.015	<0.001	0.015[2]	0.944
20:3*n-6**	0.21	0.020	0.15	0.018	0.16	0.017	0.32	0.017	0.23	0.030	0.26	0.022	<0.001	0.002[3]	0.750
20:3*n-3**	0.04	0.007	0.04	0.006	0.04	0.006	0.05	0.006	0.07	0.010	0.07	0.008	<0.001	0.145	0.071
20:4*n-6**	1.29	0.166	0.95	0.151	1.08	0.148	2.16	0.146	1.38	0.254	1.99	0.19	<0.001	0.015[4]	0.391
22:4*n-6**	0.22	0.029	0.18	0.026	0.19	0.026	0.40	0.025	0.28	0.044	0.34	0.032	<0.001	0.046[4]	0.508
Others*	2.47	0.212	2.04	0.193	2.07	0.190	3.24	0.186	2.37	0.326	3.09	0.239	<0.001	0.030[4]	0.370
Fatty acid partial sums															
SFA*	40.3	0.45	41.4	0.41	41.3	0.40	37.3	0.39	39.8	0.69	38.8	0.51	<0.001	0.001[1]	0.393
MUFA*	46.8	0.77	47.7	0.71	47.7	0.69	45.1	0.68	46.1	1.19	45.4	0.87	0.009	0.517	0.926
PUFA*	10.4	0.65	8.87	0.595	8.93	0.584	14.4	0.57	11.7	1.00	12.7	0.73	<0.001	0.009[3]	0.736
n-6 PUFA*	9.99	0.644	8.49	0.588	8.56	0.577	13.9	0.57	11.2	0.99	12.2	0.73	<0.001	0.010[4]	0.731
n-3 PUFA*	0.41	0.020	0.38	0.018	0.37	0.018	0.49	0.017	0.46	0.031	0.44	0.022	<0.001	0.074	0.981
Fatty acid ratios															
PUFA/SFA*	0.26	0.019	0.21	0.017	0.22	0.017	0.39	0.017	0.29	0.029	0.33	0.021	<0.001	0.003[3]	0.423
n-6/n-3*	24.2	1.54	22.1	1.41	23.2	1.38	28.3	1.36	25.0	2.64	28.0	1.74	0.006	0.280	0.862

[a,b] Within a row, means with different superscript letters differ, $P<0.05$.

SFA = 12:0+14:0+16:0+17:0+18:0+20:0; MUFA = 16:1*c*7+16:1*c*9+18:1*t*+18:1*c*9+18:1*c*11+20:1*c*11; PUFA = 18:2*n-6*+18:3*n-3*+20:2*n-6*+20:3*n-6*+20:3*n-3*+20:4*n-6*+22:4*n-6*; *n-6* PUFA = 18:2*n-6*+20:2*n-6*+20:3*n-6*+20:4*n-6*+22:4*n-6*; *n-3* PUFA = 18:3*n-3*+20:3*n-3*.

[1] Control<RPDL, Control<RPD, RPDL=RPD; [2] Control=RPDL, Control>RPD, RPDL=RPD; [3] Control>RPDL, Control>RPD, RPDL=RPD; [4] Control>RPDL, Control=RPD, RPDL=RPD.

* Variable adjusted for genotype × diet × IMF interaction. † Variable adjusted for IMF. ‡ Variable adjusted for genotype × IMF interaction.

Table 3.4 - Effect of the reduced protein diets equilibrated (RPDL) and not equilibrated (RPD) for lysine levels on backfat thickness P_2 (mm), total fatty acids (% fat), fatty acid composition (% total fatty acids), partial sums of fatty acids and related ratios in subcutaneous adipose tissue of Alentejano and crossbred pig genotypes.

	Alentejana						Crossbred						Significance level		
	Control		RPDL		RPD		Control		RPDL		RPD		Genotype	Diet	Genotype × diet
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE			
P_2 backfat thickness	28.0	1.33	32.0	2.02	29.8	1.33	14.7	0.97	14.8	1.14	15.8	1.74	<0.001	0.318	0.378
Total fatty acids	55.3	0.97	56.7	0.68	59.0	0.78	52.4	1.68	52.7	1.31	54.5	1.25	<0.001	0.049[1]	0.819
Fatty acid composition															
12:0	0.05	0.004	0.06	0.003	0.06	0.003	0.06	0.004	0.07	0.004	0.06	0.004	0.093	0.342	0.177
14:0	1.42[ab]	0.023	1.43[ab]	0.017	1.48[b]	0.024	1.41[ab]	0.038	1.46[ab]	0.044	1.35[a]	0.033	0.180	0.591	0.038
16:0[*]	25.5[bc]	0.29	25.9[bc]	0.28	26.4[c]	0.26	24.1[a]	0.26	24.9[ab]	0.26	23.9[a]	0.26	<0.001	0.146	0.002
16:1*c*7	0.22	0.006	0.20	0.009	0.19	0.011	0.30	0.010	0.28	0.009	0.28	0.009	<0.001	0.008[2]	0.841
16:1*c*9	1.56	0.067	1.59	0.092	1.76	0.064	1.77	0.091	1.80	0.129	1.63	0.081	0.171	0.951	0.058
17:0[†]	0.38	0.025	0.29	0.015	0.28	0.012	0.47	0.042	0.47	0.024	0.48	0.074	<0.001	0.294	0.252
18:0[‡]	15.8	0.46	16.0	0.47	15.8	0.46	14.8	0.46	14.8	0.457	15.2	0.458	0.061	0.672	0.885
18:1*t*[§]	0.17	0.012	0.21	0.021	0.17	0.010	0.23	0.019	0.18	0.019	0.20	0.018	0.226	0.108	0.148
18:1*c*9	34.3	0.63	34.4	0.44	34.9	0.66	30.4	0.53	30.4	0.39	31.9	0.68	<0.001	0.181	0.654
18:1*c*11[*]	8.08	0.423	7.81	0.404	7.27	0.382	7.45	0.385	7.20	0.383	8.00	0.385	0.602	0.808	0.141
18:2*n-6*[*]	9.81	0.233	9.08	0.226	8.87	0.193	15.3	0.452	14.6	0.30	13.4	0.624	<0.001	0.005[3]	0.434
18:3*n-3*[*]	0.80	0.019	0.72	0.019	0.70	0.019	1.05	0.032	0.96	0.021	0.88	0.045	<0.001	<0.001[2]	0.537
20:0	0.21	0.012	0.23	0.010	0.22	0.009	0.18	0.009	0.18	0.007	0.22	0.017	0.003	0.077	0.222
20:1*c*11	1.01[b]	0.033	1.09[b]	0.056	1.01[b]	0.047	0.77[a]	0.049	0.67[a]	0.021	0.89[ab]	0.095	<0.001	0.501	0.032
20:2*n-6*[*]	0.51[a]	0.021	0.51[a]	0.020	0.48[a]	0.019	0.73[c]	0.019	0.62[b]	0.019	0.68[bc]	0.019	<0.001	0.007	0.017
20:3*n-6*[*]	0.04[a]	0.004	0.04[ab]	0.004	0.05[ac]	0.005	0.08[d]	0.007	0.06[cd]	0.003	0.06[bc]	0.004	<0.001	0.110	0.001
20:3*n-3*	0.12[a]	0.004	0.14[a]	0.006	0.11[a]	0.006	0.17[b]	0.009	0.13[a]	0.008	0.14[ab]	0.011	0.003	0.020	0.002
22:4*n-6*[*]	0.07	0.011	0.08	0.007	0.07	0.005	0.25	0.019	0.21	0.011	0.24	0.024	<0.001	0.365	0.217
Others	0.29	0.015	0.25	0.009	0.25	0.021	0.42	0.042	0.38	0.024	0.34	0.046	<0.001	0.214	0.742
Fatty acid partial sums															
SFA[*]	43.5	0.67	44.0	0.64	44.2	0.61	41.1	0.61	42.5	0.61	41.3	0.61	<0.001	0.368	0.495
MUFA	45.3	0.68	45.2	0.48	45.2	0.56	41.0	0.63	40.6	0.57	43.0	0.89	<0.001	0.194	0.188
PUFA[*]	11.3	0.26	10.6	0.25	10.3	0.22	17.5	0.49	16.5	0.33	15.4	0.69	<0.001	0.003[2]	0.480
n-6 PUFA[*]	10.4	0.24	9.72	0.230	9.47	0.201	16.3	0.46	15.5	0.31	14.4	0.64	<0.001	0.004[2]	0.479
n-3 PUFA[*]	0.92	0.018	0.86	0.021	0.81	0.026	1.22	0.034	1.08	0.025	1.02	0.047	<0.001	<0.001[2]	0.249
Fatty acid ratios															
PUFA/SFA[*]	0.26	0.010	0.24	0.007	0.23	0.007	0.43	0.016	0.39	0.011	0.37	0.018	<0.001	0.006[2]	0.611
n-6/n-3	11.3	0.16	11.4	0.11	11.7	0.22	13.4	0.19	14.3	0.19	14.1	0.17	<0.001	0.005[1]	0.058

[a-d] Within a row, means with different superscript letters differ, $P<0.05$.

SFA = 12:0+14:0+16:0+17:0+18:0+20:0; MUFA = 16:1*c*7+16:1*c*9+18:1*t*+18:1*c*9+18:1*c*11+20:1*c*11; PUFA = 18:2*n-6*+18:3*n-3*+20:2*n-6*+20:3*n-6*+20:3*n-3*+22:4*n-6*; *n-6* PUFA = 18:2*n-6*+20:2*n-6*+20:3*n-6*+22:4*n-6*; *n-3* PUFA = 18:3*n-3*+20:3*n-3*.

[1] Control<RPDL, Control<RPD, RPDL=RPD; [2] Control>RPDL, Control>RPD, RPDL=RPD; [3] Control=RPDL, Control>RPD, RPDL=RPD;.

[*] Variable adjusted for genotype × diet × P_2 interaction. [†] Variable adjusted for genotype × P_2 interaction. [‡] Variable adjusted for P_2. [§] Variable adjusted for diet × P_2 interaction.

percentage of SAT weight), a significant effect of genotype (P<0.001), with the highest values for Alentejano pigs was observed. In contrast to backfat thickness, the percentage of total fatty acids in SAT was higher (P=0.049) under the RPD by 2-4%, when compared with the control diet.

The most abundant fatty acids in SAT were 18:1*c*9 (30-35% of total FAME), 16:0 (24-26%), 18:0 (15-16%), 18:2*n-6* (9-15%) and 18:1*c*11 (7-8%), in all the groups investigated. The genotype and diet interaction influenced six fatty acids, including the major SFA 16:0. The genotype affected seven of the eighteen fatty acids identified and the "others" detected fatty acids. The proportions of the major fatty acids 18:1*c*9 (P<0.001) and 18:2*n-6* (P<0.001) were the highest in Alentejano and crossbred pigs, respectively. In SAT, the diet affected only three fatty acids. The proportions of 16:1*c*7, 18:2*n-6* and 18:3*n-3* were lower in the RPD than in the control diet.

All partial sums of fatty acids and both fatty acid ratios (PUFA/SFA and *n-6*/*n-3*) were strongly affected (P<0.001) by genotype (Table 3.4). As a consequence of the genotype effect on individual fatty acids, the partial sums of SFA and MUFA were higher in Alentejano animals, whereas PUFA, *n-6* PUFA, *n-3* PUFA and both fatty acid ratios were higher in crossbred pigs. The RPD decreased PUFA (P=0.003), *n-6* PUFA (P=0.004), *n-3* PUFA (P<0.001) and PUFA/SFA ratio (P=0.006) when compared with the control diet. In contrast, the *n-6*/*n-3* ratio was increased under the RPD (P=0.005) when compared with the control diet.

3.3.3. Gene expression levels of muscle and subcutaneous adipose tissue

The results previously described demonstrate different responses of the *longissimus lumborum* muscle and SAT under RPD in the crossbred pigs, but not in the Alentejana breed. In order to investigate the mechanism underlying the genotype- and tissue-specific effects of the diets, an assessment of expression of key genes associated with lipid metabolism was carried out. The expression levels of ten key genes controlling lipid metabolism has been analysed in *longissimus lumborum* muscle and SAT, and the results are presented in Figures 3.1 and 3.2, respectively.

The expression patterns of the genes in *longissimus lumborum* muscle were similar (P>0.05) across all dietary treatments, with an exception for *PPARγ* (P=0.016) (Figure 3.1). The *PPARγ* mRNA levels were higher in the crossbred pigs when compared with the Alentejana breed. The relative expression levels of the genes investigated in the *longissimus lumborum* muscle was not affected by the dietary protein content (P>0.05). However, an interaction between genotype and diet (P=0.018) was observed for *SCD* mRNA in muscle, with the *SCD* expression increased under the RPD in crossbred animals but not in Alentejano pigs.

In SAT, relative *CEBPα* (*P*<0.001), *CRAT* (*P*=0.01), *PPARα* (*P*=0.008) and *SCD* (*P*<0.001) mRNA levels were higher in Alentejano animals when compared with the crossbred pigs (Figure 3.2). In contrast to genotype, diet did not affect the expression level of any of the genes investigated in SAT. There was a genotype and diet interaction for the mRNA levels of *ACACA* (*P*=0.044), *FABP4* (*P*<0.001), *FASN* (*P*=0.049) and *LPL* (*P*=0.009) genes in SAT. The expression level of *ACACA* and *LPL* genes were down- and up-regulated, respectively, by the RPD in the Alentejano pigs. However, these variables were not affected in the crossbred animals. The mRNA levels of *FABP4* were increased under the RPD diet in crossbred pigs. Finally, the ratio between muscle and SAT *LPL* gene expression (muscle/SAT ratio) was higher (*P*=0.045) in crossbred pigs than in Alentejano animals.

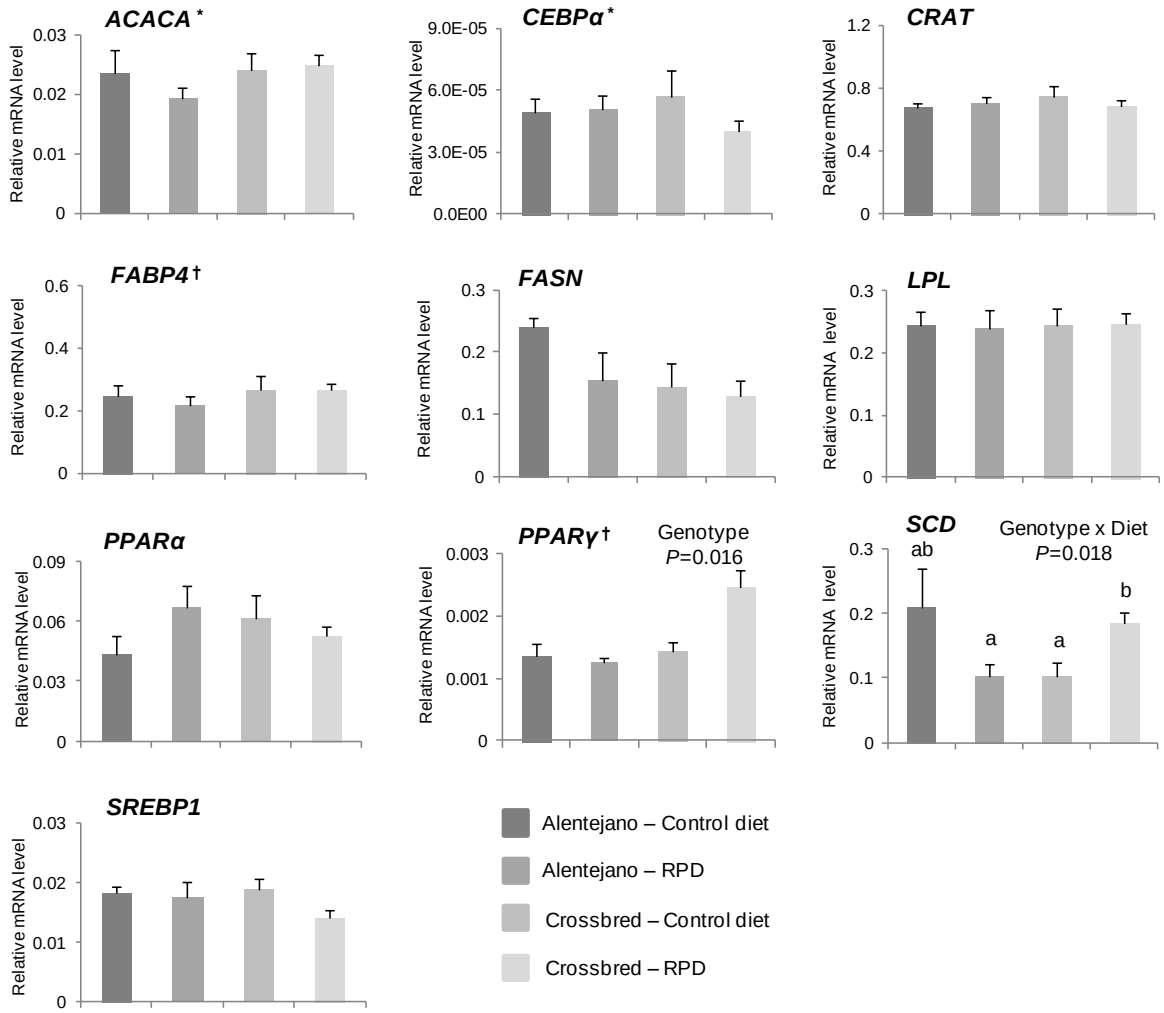

Figure 3.1 - Effect of the reduced protein diet not equilibrated for lysine level (RPD) on gene expression levels in *longissimus lumborum* muscle of Alentejano and crossbred pig genotypes. Mean ± SE without common letter differ significantly. "Genotype" and "Genotype × Diet" mean significant effect of genotype or interaction between genotype and diet, respectively. * Variable adjusted for diet × IMF interaction. † Variable adjusted for genotype × IMF interaction.

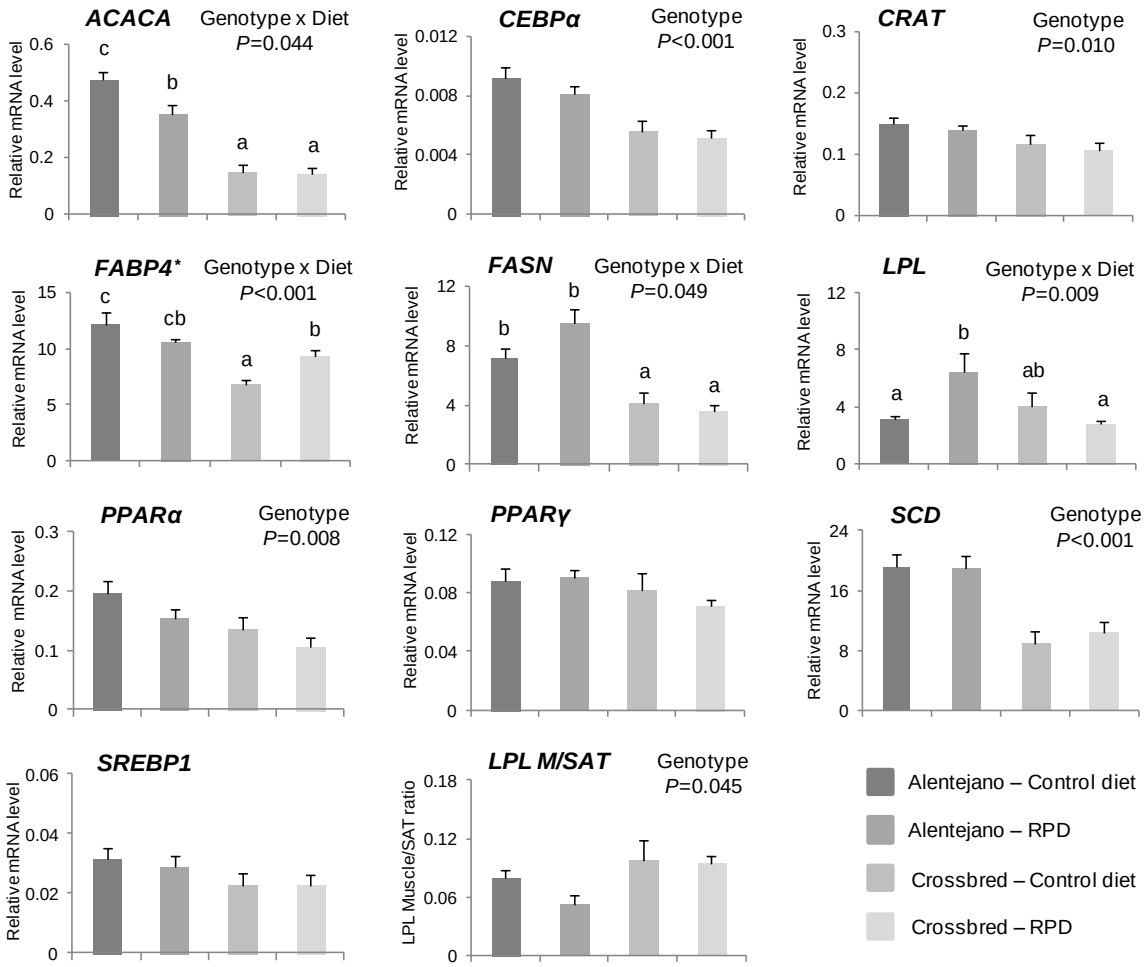

Figure 3.2 - Effect of the reduced protein diet not equilibrated for lysine level (RPD) on gene expression levels in subcutaneous adipose tissue of Alentejano and crossbred pig genotypes. Mean ± SE without common letter differ significantly. "Genotype" and "Genotype × Diet" mean significant effect of genotype or interaction between genotype and diet, respectively. * Variable adjusted for genotype × diet × P_2 interaction.

3.3.4. Correlation between fatty acid composition and gene expression levels

The correlation coefficients (r) between fatty acid composition and gene expression levels, adjusted with IMF as covariate for *longissimus lumborum* muscle and with P_2 backfat thickness as covariate for SAT, are shown in Table 3.5. In *longissimus lumborum* muscle, the 16:1*c*9 (P<0.001), MUFA (P<0.01), *PPARγ* (P<0.01)*, PPARα* (P<0.05) and *FABP4* (P<0.01) were positively and moderately correlated (0.7≥r≥0.3) with *CEBPα.* The 18:2*n*-6, and PUFA were positively correlated with *CRAT*, while MUFA was negatively correlated with the same gene. The *LPL* and *SCD* genes were not correlated with any fatty acid.

In SAT, 18:1*c*9 and MUFA were positively and moderately correlated with most of the genes, in contrast to 18:2*n-6* and PUFA, which were negatively correlated. *SCD* was positively correlated with the 18:1*c*9 (P<0.001) and MUFA (P<0.001) and negatively correlated with 18:2*n-6* (P<0.001) and PUFA (P<0.001).

Table 3.5 - Pearson´s correlation coefficients among total fatty acids (g/100 g subcutaneous adipose tissue), fatty acid composition (% total fatty acids) and gene expression levels (relative mRNA level) in *longissimus lumborum* muscle and subcutaneous adipose tissue of Alentejano and crossbred pig genotypes.

		16:0	16:1c9	18:0	18:1c9	18:1c11	18:2n-6	SFA	MUFA	PUFA	*SREBP1*	*SCD*	*PPARγ*	*PPARα*	*LPL*	*FASN*	*FABP4*	*CRAT*	*CEBPα*	*ACACA*
Longissimus lumborum muscle	*ACACA*	-0.17	0.23	-0.24	-0.24	0.44**	0.07	-0.24	-0.04	0.10	0.18	0.63***	0.63***	0.38*	0.33*	-0.07	0.61***	0.15	0.29	
	CEBPα	0.01	0.50***	-0.26	0.25	0.30	-0.37*	-0.18	0.43**	-0.33*	0.13	0.12	0.45**	0.34*	0.28	-0.21	0.47**	0.13		
	CRAT	-0.16	-0.02	-0.12	-0.38*	-0.06	0.40**	-0.18	-0.48**	0.43**	0.12	-0.08	-0.03	-0.21	0.36*	0.28	-0.04			
	FABP4	-0.16	0.30	-0.23	-0.03	0.38*	-0.07	-0.19	0.25	-0.03	0.23	0.44**	0.57***	0.30	0.56***	-0.03				
	FASN	0.23	-0.20	0.39*	-0.08	-0.17	-0.00	0.37*	-0.17	-0.03	0.02	0.19	-0.14	-0.72***	-0.04					
	LPL	-0.09	-0.06	-0.10	-0.08	-0.07	0.17	-0.05	-0.11	0.17	0.22	0.27	0.14	0.25						
	PPARα	0.02	0.31*	-0.23	-0.00	0.24	-0.07	-0.10	0.20	-0.07	0.17	0.17	0.33*							
	PPARγ	-0.23	0.48*	-0.40*	-0.04	0.60***	-0.07	-0.34*	0.30	-0.04	-0.18	0.34*								
	SCD	0.20	-0.08	0.17	0.16	-0.12	-0.18	0.23	0.10	-0.17	0.10									
	SREBP1	-0.06	-0.04	-0.01	-0.05	0.02	0.03	-0.06	-0.08	0.03										
Subcutaneous adipose tissue	TFA	0.17	0.04	0.02	0.02	-0.11	-0.07	0.12	0.00	-0.06	0.09	0.00	-0.23	-0.08	0.02	-0.03	-0.09	-0.26	-0.16	-0.09
	ACACA	0.08	-0.11	0.23	0.61***	-0.22	-0.65***	-0.07	0.41**	-0.65***	0.55***	0.84***	0.53***	0.69***	0.22	0.69***	0.63***	0.60***	0.75***	
	CEBPα	0.05	-0.20	0.08	0.64***	-0.19	-0.58***	-0.07	0.53***	-0.58***	0.62***	0.79***	0.39*	0.76***	0.25	0.58***	0.52***	0.61***		
	CRAT	0.06	0.23	-0.21	0.48**	-0.08	-0.38*	-0.16	0.50**	-0.38*	0.53***	0.64***	0.50**	0.61***	0.32*	0.61***	0.40*			
	FABP4	-0.37*	-0.26	0.11	0.64***	0.14	-0.61***	-0.50***	0.57***	-0.61***	0.20	0.51***	0.18	0.34*	-0.09	0.40*				
	FASN	0.33*	-0.07	0.27	0.63***	-0.47**	-0.62***	0.16	0.33*	-0.62***	0.37*	0.71***	0.33*	0.56**	0.39*					
	LPL	0.29	0.20	-0.07	0.33*	-0.49**	-0.21	0.19	0.21	-0.21	0.05	0.18	0.09	0.20						
	PPARα	0.02	-0.06	0.01	0.40**	-0.08	-0.36*	-0.07	0.34*	-0.36*	0.73***	0.76***	0.52***							
	PPARγ	0.16	0.17	0.07	0.17	-0.14	-0.21	0.05	0.07	-0.22	0.20	0.41**								
	SCD	0.05	-0.03	0.10	0.65***	-0.17	-0.70***	-0.06	0.56***	-0.70***	0.76***									
	SREBP1	0.05	-0.06	-0.02	0.27	0.13	-0.37*	-0.03	0.40*	-0.36*										

* $P<0.05$, ** $P<0.01$, *** $P<0.001$.

3.4. Discussion

The increased IMF obtained in the present study for growing crossbred (lean) pigs fed a 25% RPD (17.5 *vs.* 13.1% of crude protein) and not equilibrated for lysine, is in agreement with several previous studies using a range of protein concentrations (*e.g.* 20 *vs.* 16% (Da Costa *et al.* (2004); 21 *vs.* 18% (Doran *et al.* (2006)). However, whether the muscle lipogenic response was due to the reduction of dietary protein *per se*, decrease of dietary lysine level or both remains to be established. Alonso *et al.* (2010) observed an increase in IMF content (from 1.8 to 2.6% in the muscle) under the RPD (from 17 to 15%) but with similar dietary lysine contents (0.8%). In contrast, in our previous study in the traditional Bizaro pig breed (unpublished results), where we tested the effect of the reduction of dietary protein from 17% to 14% at constant lysine levels, no significant increase of IMF was obtained. This is not in line with reports of other authors, who observed a negative relationship between dietary lysine and IMF content (Hyun *et al.*, 2007). Furthermore, D'Souza *et al.* (2008) reported that pigs fed a diet with a 15% reduced lysine:energy ratio in the diet during the growing phase had higher IMF levels. This discrepancy of data might be due to the use of different pig genotypes. In fact, based on studies with two modern (Duroc and Large White breeds) and two traditional (Berkshire and Tamworth breeds) pig genotypes, Wood *et al.* (2004) suggested that the mechanisms regulating fat partitioning are genotype-specific. Therefore, it was important to undertake a comprehensive study on the effect of both low dietary protein and lysine levels on fat partitioning in diverse pig genotypes.

The present study addressed the aforementioned aspects and demonstrated that the only dietary treatment that increased IMF in *longissimus lumborum* muscle was the RPD and not the RPDL (RPD equilibrated for lysine) in crossbred pigs. Thus, the present results clearly indicate that it is the reduction of lysine availability in the diet that promotes IMF deposition in lean pig genotypes. An additional important finding of the present study is that the responses to the dietary treatments (reduction of protein or lysine) depended on the pig genotype. Thus, the IMF of Alentejana purebred (fatty) pigs in the control group was 155% higher than that of the commercial crossbred (lean pigs). In contrast to the crossbred animals, Alentejano animals did not respond to any dietary treatment.

It is well known that lysine is often the limiting amino acid for the growing rate of pigs fed cereal-based diets (NRC, 1998) and that low dietary protein and lysine levels limit protein synthesis and increase the energy available for fat deposition, with the consequent increase in IMF (Teye *et al.*, 2006). The Alentejana breed, which is similar to the Spanish Iberian breed, has a low capacity for lean tissue deposition (Garcia-Valverde *et al*., 2008) and thus lower dietary lysine requirements. Therefore, the absence of effects of RPD in Alentejana breed pigs is possibly due to the fact that lysine did not limit protein deposition.

Another possible explanation for the distinct response of the two pig genotypes to the RPD in the present experiment might be the genotype-specific expression of key lipogenic enzymes. In fact, it has been previously demonstrated that the expression of lipogenic enzymes, mainly SCD, have a critical impact on IMF deposition in pigs (Guo *et al.*, 2011). In the present study, *SCD* gene expression was increased under the RPD in crossbred but not in Alentejano pigs. In addition, the expression of the key transcription factor controlling lipid metabolism, *PPARγ*, showed a similar trend. This is consistent with the findings of Guo *et al.* (2011), who observed an increase of the *PPARγ* mRNA levels in the muscle, but not in SAT, of crossbred pigs fed high-energy low-protein diets. The present study did not find any significant effect of diet on the mRNA level of other key genes controlling fatty acid deposition, such as *ACACA*, *CEBPα*, *CRAT*, *FABP4*, *FASN*, *LPL*, *PPARα* and *SREBP1*. The
present results are consistent with the findings of Gondret & Lebret (2002), who described that *ACACA* activity in the *longissimus muscle* of pigs does not respond to feeding manipulation, including protein- and energy-restricted diets. In contrast, Damon *et al.* (2006) reported an association between IMF and FABP4 in pigs, but his study was focused on protein expression, whilst the present study investigated the mRNA content. In fact, it is well known that changes in protein expression are not always preceded by changes in mRNA expression (Gygi, Rochon, Franza & Aebersold, 1999).

The presented study showed that the RPD increased 16:0 and SFA proportions and reduced the proportions of PUFA in the *longissimus lumborum* muscle of both pig genotypes. These results are in agreement with those reported by Teye *et al.* (2006), who observed that low protein and lysine diets (21% and 1% *vs.* 18% and 0.7% of protein and lysine, respectively) in Duroc × Large White × Landrace crossbred pigs decreased total PUFA proportions. This effect could be a result of the distinct distribution of fatty acids between triacylglycerols (richer in SFA and MUFA) and phospholipids (richer in PUFA) and the increasing proportion of triacylglycerols with increasing IMF content (Ntawubizi, Raes, Buys & De Smet, 2009). Although the increased *SCD* mRNA expression in crossbred pigs fed the RPD suggests an enhanced SCD activity, the 18:1*c*9 proportion did not confirm this hypothesis. In addition, it was previously proposed by Doran *et al.* (2006) that RPD increased the IMF in pigs due to both the activation of protein expression and increased activity of SCD. Furthermore, Ntawubizi *et al.* (2009) found that IMF content was positively related to SCD and elongase activities in the *longissimus* muscle. It is well known that in monogastric animals, fatty acid composition can be strongly influenced by dietary factors. However, the dietary factors can be diluted by *de novo* SFA and MUFA biosynthesis, thus resulting in a decline of the PUFA/SFA ratio with increasing fat deposition (De Smet *et al.*, 2004). The *n-3* PUFA proportions in the present study were very low, which could be explained by low levels of *n-3*

fatty acids in cereal-based diets and is very undesirable from a human nutrition perspective (Schmid, 2011).

As expected, in the present study, backfat thickness was higher in the Alentejano (fatty) pigs than in the crossbred (lean) animals. Moreover, the content of total fatty acids in SAT, expressed on a tissue weight basis, was also higher in the Alentejano pigs. FABP4 protein is known to be responsible for the transport of fatty acids in adipocytes and its content is associated with backfat thickness (Michal, Zhang, Gaskins & Jiang, 2006) and IMF content (Damon *et al.*, 2006). Furthermore, Hocquette *et al.* (2010) suggested that FABP4 protein can be used as a marker of adipocyte number in tissues. The present study showed that *FABP4* mRNA level in SAT was 40% greater in the control group of the Alentejana breed when compared with the crossbred pigs. This is in agreement with the greater backfat thickness of the carcasses of Alentejano pigs. In addition, the up-regulation of *FABP4* gene in crossbred pigs fed with the RPD, when compared with the control crossbred pigs, is consistent with the higher content of total fatty acids in the SAT. Interestingly, the findings were different in the muscle. In spite of the higher level of IMF in Alentejano pigs, and increased IMF under the RPD in the crossbred pigs, the *FABP4* mRNA expression was not affected either by genotype or the diet. The genotype differences in the SAT fatty acid content and composition reported in the present study may be explained by a higher expression of the genes controlling lipogenesis (*ACACA*, *FASN* and *SCD*) and expression of the transcription factor *CEBPα*. This suggestion is in line with results of De Pedro (2001), who reported that carcasses of Iberian pigs, a genotype similar to Alentejana purebred, have higher fatty acid contents than commercial crossbred genotypes in the SAT. The higher *FASN* expression level in SAT of Alentejano pigs, when compared with the crossbred animals, observed in the present study, is consistent with the higher 16:0 proportion in SAT of Alentejano pigs, as 16:0 is the end product of *de novo* synthesis of SFA.

In addition to the genotype-specific response to the RPD, another important finding of the present study is the tissue-specific effect of the same diet. The crossbred pigs demonstrated a large increase in fat content in muscle (55%), with only a small increase in total fatty acids content in SAT (4%) under the RPD. Furthermore, tissue-specific responses to RPD were also observed in the mRNA expression patterns for *ACACA*, *FASN* and *SCD*. This is in line with the results of Doran *et al.* (2006), who observed significant differences in responses of muscle and SAT fatty acid composition and SCD to a RPD. This was tentatively explained by tissue-specific expression of SCD isoforms. In adipose tissue, which is the main site for *de novo* fatty acid synthesis in pigs (O'Hea & Leveille, 1969; Dodson *et al.*, 2010), SCD activity was apparently not affected by the RPD because the percentages of 16:1*c*9 and 18:1*c*9 and the *SCD* mRNA levels did not change. Thus, it is very unlikely that SCD activity could increase with low protein diets in this tissue. In addition, intramuscular adipose tissue, the

last fat depot to develop, may respond to dietary conditions in a different manner from other fat sites (Gondret & Lebret, 2002).

Previous gene expression profiling and proteomics studies suggested that pathways involved in lipid and energy metabolism are clearly down-regulated in intramuscular adipocytes when compared with fat cells from other depots (Gardan *et al.*, 2008; Liu *et al.*, 2009; Cánovas, Quintanilla, Amills & Pena, 2010). In the present study, regardless of the genotype or diet, mRNA levels of *ACACA*, *FASN*, *FABP4*, *PPARγ*, *LPL*, *CEBPα*, *SCD* and *SREBP1* were higher in SAT than in muscle. Also, major fatty acids and partial sums of fatty acids were much more correlated with the expression level of key lipogenic enzymes and transcription factors in SAT than in muscle. Although muscles contain a relatively low proportion of adipocytes, some authors found that mRNA levels of genes related to lipid metabolism were lower in intramuscular adipocytes than in subcutaneous adipocytes (Zhou *et al.*, 2010). However, in the present study, the expression level of *CRAT* was lower in SAT than in muscle, thus suggesting a higher activity of β-oxidation of fatty acids in muscle than in SAT. Although CEBPα, PPARγ and SREBP1 are key regulators of adipogenesis, it was suggested that SREBP1 is a transcription factor induced during the early stages of adipogenesis, inducing the expression of CEBPα and PPARγ only in the later phases of fat deposition (Payne *et al.*, 2009). This may explain the absence of genotype differences or responses of this adipogenic factor to dietary treatment in the present study. This suggestion is in line with the findings by Ding, Schinckel, Weber & Mersmann (2000), who demonstrated that SREBP1 mRNA expression in adipose tissue does not differ between Newsham-sired and Duroc-sired pigs, suggesting that genetic selection does not affect expression of the aforementioned gene.

LPL is a rate-limiting enzyme responsible for hydrolysis of circulating triacylglycerols carried out in VLDL and chylomicrons and is, generally, produced primarily by muscle and mature adipocytes (Fielding & Frayn, 1998). Therefore, LPL modulates partitioning of fatty acids between oxidation in skeletal muscle and storage in white adipose tissue (Tan *et al.*, 2011). The results of the present study showed that *LPL* mRNA was expressed at higher levels in SAT, when compared with the muscle (LPL muscle/SAT ratios <0.1). This suggests that circulating fatty acids were mainly used for storage in SAT. In addition, the higher *LPL* muscle/SAT ratios in the crossbred pigs, when compared with the Alentejano animals, indicate a lower storage capacity of SAT in the crossbred pigs. Interestingly, plasma concentrations of triacylglycerols (318 *vs.* 388 mg/l, P=0.002, for the control diet and RPD, respectively) and NEFA (46 *vs.* 68 µmol/l, P=0.038, for the control diet and RPD, respectively) in Alentejano pigs had a similar pattern to that of *LPL* gene expression in SAT, i.e. with increased values for the RPD when compared with the control diet. Therefore, it is possible that once NEFA are released from VLDL by the action of LPL, they are taken up

mainly by the adipocytes of SAT, thus increasing the mass of this fat depot in Alentejana breed pigs.

3.5. Conclusions

To the best of our knowledge, the present study is the first report that demonstrated that RPD increase the IMF content in lean but not in fatty pig genotypes. Furthermore, the present results strongly suggest that the increased IMF deposition in lean pig genotypes is due to a limitation of lysine level in the diets. Analyses of mRNA expression suggest that the genotype-specific effect of the RPD on IMF content is mediated via up-regulation of the expression of lipogenic enzyme SCD and the adipogenic transcription factor *PPARγ*. The muscle fatty acid composition was more affected in lean than in fatty pigs under the RPD, which may be reflected in the change in the TAG/phospholipid ratio, as result of the increased IMF in the former genotype.

Furthermore, the present results indicate that feeding a RPD does not change the backfat thickness, but results in an increase in total fatty acid content in both lean and fatty pig genotypes. When backfat thickness was compared between the control groups of both genotypes, it was established that the higher backfat thickness of fatty pigs, when compared with the lean ones, is associated with higher mRNA levels of the key lipogenic enzymes and transcription factors (*ACACA*, *CEBPα*, *FASN* and *SCD*). Therefore, we can conclude that the fatty acid composition of SAT seems to be more affected by the genotype than by the diet, under these experimental conditions.

Overall, the results strongly suggest that adipogenesis and lipogenesis are regulated differently in the *longissimus lumborum* muscle and SAT of pigs, and that this modulation is genotype specific. These findings could help in the development of effective genotype-specific feeding strategies in order to improve fat partitioning in pigs. This insight into the molecular mechanisms underlying regulation of the amount and composition of IMF in pigs may contribute to the development of strategies to satisfy consumers' expectations and to enhance the competitiveness of the meat industry.

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Chapter 4

THE COMBINATION OF ARGININE AND LEUCINE SUPPLEMENTATION OF REDUCED CRUDE PROTEIN DIETS IN PIGS INCREASES MEAT EATING QUALITY

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Contribution of Marta S. Madeira to this article:
Marta S. Madeira collaborated in animal experiment, tissues sampling and did the meat quality parameters, laboratory analysis, data processing and statistical analysis. In addition, Marta S. Madeira integrated the trained sensory analysis panel and help in the interpretation of the results and writing of the manuscript.

The combination of arginine and leucine supplementation of reduced crude protein diets in pigs increases meat eating quality

Abstract

Fifty four entire male pigs from a commercial crossbred (Duroc × Pietrain × Large White × Landrace) were used to investigate the effect of dietary arginine supplementation, crude protein reduction and crude protein reduction with leucine addition on growth performance, carcass traits and meat quality. Pigs weighing 58.9 ± 1.59 kg body weight were randomly assigned to one of six treatments (*n*=54). The treatments followed a 2 × 3 factorial arrangement, with arginine supplementation with two levels (0 vs. 1%) and the basal diet with three levels: normal crude protein diet (16% crude protein, NPD), reduced crude protein diet (13% curde protein, RPD) and reduced crude protein diet with leucine addition to 2.0% (RPDL). Pigs were slaughtered at 91.7 ± 1.6 kg body weight. Dietary arginine supplementation had no effect (*P*>0.05) on IMF content but produced meat off-flavour and, under NPD, increased meat juiciness. The RPD and RPDL increased (*P*<0.01) IMF content (45-48%) but negatively affected the growth performance of animals. Moreover, RPD and RPDL increased (*P*<0.05) backfat thickness and decreased loin weight. Leucine addition in reduced crude protein diets had no effect (*P*>0.05) on IMF content, growth performance, backfat thickness and loin weight. Within pigs fed control diet, there was an increase of juiciness in RPD and RPDL, which accompanied the increase of IMF content with the low crude protein diet. RPD and RPDL increased meat deposition of 18:1*c*9, SFA, MUFA and PUFA, which were not correlated with any pork sensory trait. The main combined effect of arginine and leucine addition on RPD was an increased tenderness and overall acceptability of pork. In conclusion, it was confirmed that dietary crude protein reduction enhances pork eating quality but negatively affected growth performance and carcass characteristics of pigs. In addition, the combined impact of arginine and leucine supplementation of reduced crude protein diets in lean pig genotypes improves meat sensory quality, when compared with the effect promoted by the RPD alone.

Keywords: arginine, crude protein, intramuscular fat, leucine, pork quality, sensory panel

4.1. Introduction

Pigs reared in intensive systems have become leaner to satisfy consumers and faster growing to improve feed efficiency (Wood *et al.*, 2008). However, these changes have been made at the expense of an IMF reduction, which has well known adverse effects on pork eating quality (Hocquette *et al.*, 2010). IMF content of pork plays an important role in consumers' perceptions of cooked pork palatability (Lonergan *et al*., 2007), and it has been suggested that an IMF content between 2.5 and 3.0% is necessary for consumer acceptability of cooked pork (De Vol *et al*., 1988). However, due to a poor relationship between IMF and subcutaneous fat thickness (Wood *et al*., 2004), it is possible to increase marbling while maintaining pig leanness (improved fat partitioning), which is highly desirable for the meat industry.

It has been reported that dietary protein restriction (Doran *et al.*, 2006; Alonso *et al.*, 2010) and leucine supplementation (Hyun *et al.*, 2003 and 2007) during the growing-finishing period of pigs could enhance IMF content without increasing subcutaneous fat. Unfortunately, these studies also reported decreased feed efficiency and increased carcass fat content. However, dietary arginine supplementation has been reported as being beneficial for promoting IMF content, skeletal muscle gain, carcass lean content and improved meat quality, without undesirable effects on growth performance (Tan *et al.*, 2009; Ma *et al.*, 2010). Our group reported recently that the increased IMF promoted by dietary crude protein reduction in pig is due to lysine limitation and improves pork sensory attributes (Madeira *et al.*, 2013a, Chapter 2). To our knowledge, there are no reports of the combined impact of arginine, protein level (low lysine) and crude protein reduction plus leucine on carcass traits and pork quality. However, these different feeding strategies could have additive/interaction effects. Therefore, it was hypothesized that the increased IMF and meat eating quality induced by dietary crude protein reduction in the growing-finishing period of commercial crossbred pigs can be modulated by arginine and/or leucine supplementation, without major undesirable effects on growth rate and carcass traits.

4.2. Material and Methods

This trial was conducted in accordance with European Union standard guidelines for humane care and use of animals in experimental research (Directive 86/609/EEC).

4.2.1. Animals and experimental diets

Fifty four crossbred entire male pigs with four genetic lines (50% Large White × 50% Landrace gilts mated to 50% Duroc × 50% Pietrain boars) with a body weight of 58.9 ± 1.59 kg were selected. Before the beginning of the experiment, all animals were housed and fed with the same conventional feed management (based on starter and growth concentrates). Afterward, groups of pigs (3 pens with individual control of feed intake, containing 3 pigs each), were randomly assigned to 1 of the 6 isoenergetic diets (14 MJ ME/kg) dietary treatments. The treatments followed a 2 × 3 factorial arrangement, with arginine supplementation with two levels (0 vs 1%) and basal diet with three levels: normal crude protein diet (16.0% crude protein, NPD); reduced crude protein diet (13.0% crude protein, RPD); and reduced crude protein diet with leucine addition to 2.0% (RPDL). The diets not supplemented with arginine were supplemented with an equivalent amount of alanine (2.05%) in order to be an isonitrogenous control (Table 4.1). The amino acids were obtained from Fh Diedrichs & Ludwig Post (Mannheim, Germany). During the experiment, animals were fed individually, twice a day, using a system of gates that isolated the pigs while they were eating, and had *ad libitum* access to water. Feed offered and refusals were recorded daily for each pig in order to obtain individual feed intake.

Diet samples were collected 5 times during the trial. Diets were analyzed for DM by drying a sample at 100 ºC to a constant weight. The nitrogen content was determined by the Kjeldahl method described in AOAC (2000) and crude protein was calculated as 6.25 × nitrogen. The determination of ash and starch contents was performed according to the procedures described by AOAC (2000) and Clegg (1956), respectively. Crude fat was determined by extracting feed samples with petroleum ether, using an automatic Soxhlet extractor (Gerhardt Analytical Systems, Königswinter, Germany). Crude fiber was determined according to the procedure described by AOAC (2000). The gross energy content of diets was measured using an adiabatic bomb calorimeter (Parr 1261, Parr Instrument Company, Moline, IL). Fatty acid methyl esters of feed samples were analyzed by one-step extraction transesterification, using heptadecanoic acid (17:0) as internal standard (Sukhija & Palmquist, 1988). The amino acid composition was extracted according to the method described by AOAC (2005) and the quantification was performed by HPLC (Agilent 1100, Agilent Technologies, Avondale, PA), according to Henderson *et al*. (2000).

Table 4.1 - Ingredients and analyzed chemical composition of the experimental diets.

	Control			Arginine		
	NPD	RPD	RPDL	NPD	RPD	RPDL
Ingredients, %						
Corn	62.9	67.3	75.0	63.7	72.3	74.5
Barley	10.0	15.0	8.00	10.0	10.0	10.0
Soybean meal	18.9	10.9	9.60	16.3	7.80	7.2
Sunflower meal	1.64	0.44	-	4.56	4.66	1.98
Soybean oil	1.15	0.98	0.99	1.06	0.88	0.85
Calcium carbonate	0.73	0.73	0.71	0.72	0.70	0.71
Sodium bicarbonate	0.11	0.01	-	0.14	0.06	0.07
Vitamin-trace mineral premix[1]	0.40	0.40	0.40	0.40	0.40	0.40
Salt	0.35	0.43	0.44	0.33	0.39	0.38
Bicalcium phosphate	1.21	1.32	1.38	1.22	1.35	1.39
L-Lys	0.30	0.12	0.17	0.34	0.17	0.21
L-Met	0.06	-	-	0.06	-	-
L-Thr	0.07	-	-	0.08	-	-
L-Ala	2.05	2.05	2.05	-	-	-
L-Arg	-	-	-	1.00	1.00	1.00
L-Leu	-	0.17	1.14	-	0.17	1.17
Acid mixture[2]	0.10	0.10	0.10	0.10	0.10	0.10
Antioxidant mixture[3]	0.005	0.005	0.005	0.005	0.005	0.005
Chemical composition						
DM, %	87.5	87.7	87.8	87.7	87.7	87.9
Crude protein, %	16.0^{b}	13.1^{a}	13.1^{a}	15.9^{b}	12.9^{a}	12.7^{a}
Starch, %	38.3^{a}	42.6^{b}	42.5^{b}	38.5^{a}	42.5^{b}	43.1^{b}
Crude fat, %	3.36	3.46	3.54	3.46	3.46	3.56
Crude fiber, %	4.38^{bc}	3.22^{a}	3.06^{a}	4.66^{c}	4.20^{b}	3.36^{ab}
Ash, %	3.88^{a}	3.78^{a}	3.78^{a}	4.16^{b}	3.98^{ab}	3.80^{a}
Calcium, %	0.66	0.73	0.75	0.59	0.68	0.71
Phosphorus, %	0.49	0.51	0.52	0.51	0.52	0.52
Metabolisable energy (MJ/kg)	13.8	14.1	14.3	13.9	14.1	14.3
Lys:ME, %/MJ ME	0.061^{b}	0.033^{a}	0.031^{a}	0.051^{b}	0.030^{a}	0.030^{a}
AA composition, %						
Ala	3.13^{c}	3.25^{cd}	3.52^{d}	0.16^{a}	0.51^{b}	0.33^{ab}
Arg	1.05^{c}	0.83^{b}	0.49^{a}	1.84^{e}	1.60^{d}	1.56^{d}
Asp	0.49^{c}	0.35^{b}	0.31^{a}	0.45^{c}	0.38^{b}	0.30^{a}
Glu	2.07^{e}	1.54^{bc}	1.38^{ab}	1.82^{d}	1.59^{c}	1.34^{a}
Gly	0.43^{bc}	0.35^{a}	0.41^{ac}	0.63^{d}	0.43^{bc}	0.41^{ab}
His	2.02^{c}	1.21^{ab}	0.92^{a}	1.27^{b}	1.02^{ab}	0.90^{a}
Ile	0.45^{c}	0.32^{a}	0.38^{b}	0.50^{d}	0.35^{ab}	0.35^{ab}
Leu	1.01^{b}	0.93^{a}	1.51^{c}	0.95^{ab}	0.94^{ab}	1.74^{d}
Lys	0.84^{c}	0.47^{a}	0.45^{a}	0.70^{b}	0.43^{a}	0.43^{a}
Met	0.02^{a}	0.04^{ab}	0.07^{bc}	0.06^{b}	0.18^{d}	0.10^{c}
Phe	0.68^{d}	0.47^{c}	0.28^{ab}	0.39^{b}	0.33^{a}	0.31^{a}
Pro	0.83^{bc}	0.79^{b}	0.61^{a}	0.85^{bc}	0.96^{d}	0.89^{c}
Ser	0.81^{d}	0.67^{c}	0.61^{ab}	0.78^{d}	0.63^{bc}	0.57^{a}
Thr	0.17^{b}	0.10^{a}	0.12^{a}	0.20^{c}	0.19^{bc}	0.18^{bc}
Tyr	0.31^{d}	0.20^{bc}	0.18^{b}	0.24^{c}	0.17^{ab}	0.13^{a}
Val	0.70^{c}	0.56^{b}	0.44^{a}	0.57^{b}	0.47^{a}	0.45^{a}
Fatty acid composition, % total fatty acids						
16:0	15.1	15.2	14.9	14.9	15.1	15.0
18:0	2.87^{b}	2.48^{a}	2.60^{a}	2.64^{ab}	2.45^{a}	2.44^{a}
18:1*c*9	24.8^{a}	25.1^{a}	25.8^{b}	25.0^{a}	25.5^{b}	25.6^{b}

18:1*c*11	1.04[e]	0.97[bc]	0.98[c]	1.01[d]	0.95[ab]	0.94[a]
18:2*n-6*	52.9	53.1	52.9	53.3	53.3	53.2
18:3*n-3*	3.30[e]	3.11[c]	2.86[b]	3.21[d]	2.77[a]	2.78[a]

[a,b]Within a row, means with different superscript letters differ, $P<0.05$ (n=5).
NPD, normal crude protein diet; RPD, reduced crude protein diet; RPDL, reduced crude protein diet with Leu supplementation.
[1]Vita Tec (Tecadi, Santarém, Portugal). Provided per kilogram of diet: vitamin A, 6,000 IU; vitamin D3, 1,500 IU; vitamin E (acetate dl-alpha-tocopherol), 15 mg; vitamin B2, 0.3 mg; vitamin B12, 3.75 mg; biotin, 0.1 mg; calcium pantothenate, 12 mg; nicotinic acid, 15 mg; folic acid, 0.75 mg; choline chloride, 200 mg; Cu (cupric sulfate pentahydrate), 15 mg; Zn (zinc oxide), 100 mg; Mn (manganese oxide), 35 mg; I (potassium iodide), 0.7 mg; Co (basic cobaltous carbonate mono hydrous), 0.05 mg; Se (sodium selenite), 0.2 mg; Fe (ferrous carbonate), 80 mg; and BHT, 0.2 mg.
[2]Formic acid, propionic acid, citric acid, and calcium salts (Ultracid V Dry EU; Tecadi).
[3]Ethoxyquin, propyl gallate, and citric acid (Oxi-Nil Dry Premix; Tecadi).

4.2.2. Animal performance and muscle sampling

Throughout the experiment, pigs were weighed weekly before feeding. The ADG and G:F were calculated. Feed was withdrawn from animals 17 to 19 h before slaughter. Pigs were slaughtered at a body weight of 91.7 ± 1.61 kg at the UIPA-INIAV experimental slaughterhouse in Santarém (Portugal), following standard handling procedures and using electrical stunning before exsanguination. The HCW was recorded and carcass yield was calculated. Perirenal fat was removed and weighed. For IMF content and fatty acid composition, samples of *longissimus lumborum* muscle were collected from the right carcass side between third and fifth lumbar vertebras, immediately vacuum packed and stored at -20 ºC until analyses.

At 24 h postmortem, backfat thickness was measured in the left carcass side at the shoulder, last rib position (P_2, the most representative location), last lumbar vertebra (L6), and second sacral vertebra (S2), as described by Frederick (1972). The loin was excised from the left side of the carcass between last cervical and L6 lumbar vertebras, and weighed just before being sliced into 2.5-cm-thick chops for sensory evaluation (second to third lumbar vertebras) and shear force measurements (first lumbar vertebra). Chops were vacuum packaged, frozen and stored at -20 ºC until further analyses.

4.2.3. Meat quality traits

The pH and muscle temperature were measured in the *longissimus lumborum* muscle (L1, right carcass side) at 45 min (pH_{45} and T_{45}, respectively) and 24 h (pH_{24} and T_{24}, respectively) postmortem, using a pH meter equipped with a penetrating electrode (HI8424, Hanna Instruments, Smithfield, RI). Objective colour was measured on the cut surface of the *longissimus* muscle section 24 h postmortem, using a chromometer (Minolta CR-300; Konica Minolta, Tokyo) as previously described by Madeira *et al.* (2013a) (Chapter 2).

4.2.4. Intramuscular fat content and fatty acid composition

The IMF content was determined in fresh samples by hydrolysis with 4 M HCl, followed by Soxhlet extraction, with petroleum ether as described by AOAC (2000). To assess fatty acid composition in muscle, total lipids were extracted from lyophilized samples (approximately 250 mg), using dichloromethane:methanol (2:1, vol/vol), the method adapted from Folch & Stanley (1957). Fatty acids were converted to FAME by a combined transesterification procedure with NaOH in anhydrous methanol (0.5 M), followed by HCl:methanol (1:1 vol/vol), at 50 °C during 30 and 10 min, respectively, according to the method described by Raes *et al.* (2001). The FAME were analyzed using a gas chromatograph (HP7890A; Hewlett-Packard, Avondale, PA), equipped with a flame ionization detector (GC-FID) and a Supelcowax^{TM} 10 capillary column (30 m × 0.20 mm i.d., 0.20 µm film thickness; Supelco, Bellefonte, CA). The chromatographic conditions were as follows: the injector and detector temperature were 250 and 280 ºC, respectively, helium was used as carrier gas at a flow rate of 1.3 mL/min, and the split ratio was 1:20. The gas chromatograph oven temperature was programmed to start at 150 ºC (maintained for 11 min), followed by a 3 ºC/min ramp to 210 ºC (maintained for 30 min).

The quantification of total FAME was done using nonadecanoic acid (19:0) as internal standard and the conversion of relative peak areas into weight percentages. Fatty acids were identified on the basis of their retention times, corresponding to their FAME standard (Supelco Inc., Bellefonte, PA) and expressed as mg/100 g of muscle.

4.2.5. Free amino acids of muscle

The nitrogen content in lyophilized muscle samples was determined by the Kjeldahl method (AOAC, 2000) and crude protein was calculated as 6.25 × nitrogen.

For free amino acids determination, samples of muscle were extracted and deproteinised according to the method described by Aristoy & Toldrá (1991). One gram of *longissimus lumborum* muscle was homogenized with 0.1 N HCl (dilution 1:10) using an Ultra-Turrax T25 homogenizer (IKA Werke GmbH & Co. KG, Staufen, Germany) for 1 min at 18000 rpm. These homogenates were centrifuged at 4 ºC at 10000 × *g* for 20 min. The supernatant was filtered through filter paper and stored at -80 ºC until use. Then 200 µl of thawed sample plus 20 µl of an internal standard solution (norvaline and sarcosine, 10 nmol/µl each; Sigma-Aldrich, St. Louis, MO, USA) was deproteinised by adding 780 µl of ethanol and centrifuged at 14000 × *g* for 10 min. The supernatant was derivatised by online column derivatization using o-phthalaldehyde (OPA) for primary amino acids and 9-fluorenylmethyl chloroformate (FMOC) for secondary amino acids, according to Henderson *et al.* (2000). The derivatised

amino acids were analyzed by reversed-phase HPLC using a Gemini C18 column (150 mm × 4.60 mm, 5 µm Phenomenex, Torrance, CA, USA). The separation was performed at 40 ºC using a gradient between two solvents: 40 mM sodium phosphate at pH 7.8 (solvent A) and acetonitrile:methanol:water, 45:45:10 v/v (solvent B). The flow rate was 2 mL/min and the solvent gradient was: initial 0% B until 1.9 min, 16.2 min linear change to 57% B, 0.5 min linear change to 100% B and maintained for 3.7 min. The amino acid analysis was achieved in 22 min. The detection was monitored by fluorescence signal monitored at 450 nm for emission and 340 nm for excitation.

4.2.6. Meat lipid oxidation

Lipid oxidation in meat was assessed through the quantification of thiobarbituric acid reactive substances (TBARS), based on the spectrofotometric method of Grau, Guardiola, Boatella, Barroeta & Codony (2000). In brief, samples were homogenized in the presence of 8 mL of 5 g/100 mL aqueous trichloroacetic acid and 5 mL of 0.8 g/100 mL butylated hydroxytoluene (BHT) in hexane, and the mixture was centrifuged. The top layer was discarded, and a 2.5 mL aliquot from the bottom layer was mixed with 1.5 mL of 0.8 g/100 mL aqueous 2-thiobarbituric acid to be further incubated at 70 ºC for 30 min. Following incubation, the mixture was cooled under tap water. This method is based on the ability of malonaldehyde (MDA) to form a pink-coloured chromogen that absorbs at 532 nm in a UV/VIS Spectrophotometer (Ultrospec III, Pharmacia LKB Biochrom Ltd., Cambridge, England).

For the assessment of meat lipid stability, (antioxidant potential) the general procedure of Mercier, Gatellier & Renerre (2004) was followed, which involves the induction of oxidation in meat homogenate by ferrous iron and hydrogen peroxide, followed by the measurement of oxidized lipids. Meat homogenates were prepared by homogenising 1 g tissue in 10 mL of 100 mM sodium phosphate (pH 7.0) using an Ultra-Turrax T25 homogenizer (IKA Werke GmbH & Co. KG, Staufen, Germany) for 1 min at 20000 rpm. These homogenates were incubated with 100 µl of mixture of ferrous sulphate (0.5 mM) and hydrogen peroxide (1 mM) at 37 °C water bath for 30 min. After incubation, 2 mg of BHT was added to homogenate to stop the oxidation. Aliquots of 2 mL of homogenate were immediately frozen at -80 °C until lipid oxidation measurement by TBARS as described by Lynch & Frei (1993). Briefly, homogenate samples (0.5 mL) were incubated with 0.25 mL of 1% (w/v) 2-thiobarbituric acid in 50 mM of NaOH and 0.25 mL of 2.8% (w/v) tricloroacetic acid in boiling water bath for 10 min. The pink chromogen was extracted with 2 mL of *n*-butanol and its absorbance measured at 535 nm.

To quantify TBARS, a standard calibration curve was constructed employing 1,1,3,3 tetraethoxypropane (Fluka Neu Ulm, Germany) as precursor of MDA. The results were expressed as mg of MDA/kg of meat.

4.2.7. Shear force measurements

Frozen chops were thawed at 4 ºC (24 h) and cooked in a plate grill (65/70 FTES Electric Griddle, Modelar Catering Equipment, Italy) at 250 °C until they reached an internal temperature of 71 °C, which was monitored by an internal thermocouple (Lufft C120, München, Germany). The cooking loss was determined by calculating the difference in weight before and after thermal processing. The WBSF (kg) was measured in a texture analyzer (TA-tx2i Texture Analyser, Stable Micro Systems, Surrey, UK) as described by Madeira *et al.* (2013a) (Chapter 2).

4.2.8. Trained sensory panel analysis

For each session of trained sensory panel analysis, 9 chops were thawed and cooked, using the same conditions described for shear force measurements. All samples were trimmed of external connective tissue and cut into cores with approximately 2 × 2 × 2 cm, maintained at 60 ºC in heated plaques and tasted as soon as possible. Twelve trained panelists for pork performed the sensory analysis in 6 sessions (9 samples for each session). The panelists were selected and trained according to Cross *et al.* (1978). Samples were randomly distributed across sessions and the attributes classified were tenderness, juiciness, flavor, and overall acceptability. The scale applied in the sensory analysis was structured into 8 points as previously described by Madeira *et al.* (2013a) (Chapter 2).

4.2.9. Statistical analysis

Data were analyzed using PROC MIXED (SAS Inst., Inc., Cary, NC) considering the animal as the experimental unit and allowing for variance heterogeneity, when necessary. The model included the main effects of Arg supplementation, type of basal diet (NPD, RPD and RPDL) and their interaction as fixed effects. In order to allow an easier evaluation of the basal diet main effects, the P value of this main effect was replaced in the tables by three contrasts comparing the three levels of the basal diet: NPD *vs*. RPD, NPD *vs*. RPDL and RPD *vs*. RPDL. If the interaction was significant ($P<0.05$), multiple comparisons of least square means for traits were compared using the PDIFF, with Tukey-Kramer adjustment options of SAS. Pearson's correlation coefficients were calculated with the CORR procedure of SAS to elucidate possible associations among carcass characteristics, meat traits, and major fatty acids (expressed as g/100 g of total FA), as well as meat sensory attributes with muscle free amino acids. The correlations were considered significant at $P<0.05$.

4.3. Results

4.3.1. Growth performance and carcass traits

The results of growth performance and carcass characteristics of pigs are shown in Table 4.2. The dietary arginine supplementation had no influence on the growth parameters (P>0.05). When the level of dietary protein was reduced (RPD), an increase in ADFI was observed (P<0.05) relative to the NPD, which was not observed (P>0.05) when leucine was added to the RPD (RPDL). When compared with pigs fed NPD, the ADG was lower in pigs fed RPD (P<0.05) and RPDL (P=0.001). The G:F values were greater in pigs fed the NPD than in those fed with the RPD and RPDL.

Concerning carcass characteristics, only HCW was not affected by diet (P>0.05). However, an interaction between arginine and protein level was observed for carcass yield (P=0.017), perirenal fat (P=0.048) and S2 backfat thickness (P=0.007). The reduced protein diets (RPD and RPDL) increased the shoulder and P_2 backfat thickness (P<0.05) when compared with the pigs fed the NPD. The pigs fed the RPD (P<0.05) and RPDL (P<0.01) diets had a lower loin weight relative to the pigs fed the NPD.

4.3.2. Meat quality traits

Pork quality traits assessed in *longissimus lumborum* muscle are presented in Table 4.3. An interaction between dietary arginine and protein level (P<0.05) was observed for pH at 45 min. Regardless of the protein level, when pigs had dietary arginine supplementation, the values were similar. However, in the control pigs, the pH at 45 min was lower in the pigs fed RPD and RPDL when compared with the NPD. In addition, neither protein level nor arginine supplementation influenced temperature, colour, TBARS, lipid oxidation, shear force and cooking loss (P>0.05).

Table 4.2 - Growth performance and carcass characteristics of pigs.

Item	Control			Arginine			SEM	Significance level				
								Arg	Dietary protein level			Arg×Prot
	NPD	RPD	RPDL	NPD	RPD	RPDL			NPD *vs*.RPD	NPD *vs*.RPDL	RPD *vs*.RPDL	
Growth performance												
ADFI, kg	2.43	2.59	2.51	2.59	2.80	2.50	0.084	0.093	0.037	0.916	0.029	0.408
ADG, g	814	697	673	828	752	648	47	0.700	0.046	0.001	0.182	0.696
G:F, kg/kg	0.33	0.27	0.26	0.32	0.27	0.26	0.013	0.521	<0.001	<0.001	0.653	0.832
Carcass characteristics[1]												
HCW, kg	73.6	72.1	73.1	73.5	73.5	72.4	0.49	0.601	0.135	0.093	0.850	0.103
Carcass yield, %	81.1^{c}	78.4^{a}	79.5^{ac}	79.9^{bc}	79.4^{abc}	79.4^{ab}	0.36	0.821	<0.001	0.007	0.127	0.017
Perirenal fat, kg	0.51^{a}	0.73^{bc}	0.78^{c}	0.54^{ab}	0.88^{c}	0.70^{ac}	0.047	0.415	<0.001	<0.001	0.177	0.048
Shoulder, mm	20.6	23.3	22.6	21.2	25.8	25.8	1.60	0.109	0.026	0.044	0.822	0.708
P_2 backfat thickness, mm	17.1	18.1	19.1	15.3	20.2	20.0	1.38	0.720	0.041	0.021	0.780	0.349
L6 backfat thickness, mm	15.1	17.4	15.2	15.4	19.9	18.0	1.19	0.057	0.006	0.264	0.091	0.538
S2 backfat thickness, mm	8.18^{a}	8.71^{a}	8.89^{ab}	7.52^{a}	12.8^{c}	11.5^{bc}	0.73	0.001	<0.001	0.002	0.453	0.007
Loin weight, kg	5.51	5.08	5.10	5.36	5.26	5.20	0.103	0.614	0.013	0.007	0.817	0.238

[a-c]Within a row, means with different superscript letters differ, $P<0.05$.

NPD, normal crude protein diet; RPD, reduced crude protein diet; RPDL, reduced crude protein diet with Leu supplementation.

[1]P2, at the last rib; L6, at the last lumbar vertebra; and S2, second sacral vertebra.

Table 4.3 - Meat traits of *longissimus lumborum* muscle from pigs.

Item	Control			Arginine			SEM	Significance level				
								Arg	Dietary protein level			Arg × Prot
	NPD	RPD	RPDL	NPD	RPD	RPDL			NPD *vs.* RPD	NPD *vs.* RPDL	RPD *vs*. RPDL	
Temperature, ºC												
45 min	21.6	22.2	21.8	20.7	22.5	20.9	0.94	0.534	0.212	0.848	0.289	0.795
24 h	7.88	7.81	7.40	7.50	8.07	7.24	0.808	0.889	0.758	0.652	0.449	0.924
pH												
45 min	6.10^{b}	5.87^{a}	6.03^{a}	5.93^{ab}	6.12^{b}	6.09^{b}	0.074	0.417	0.818	0.538	0.398	0.025
24 h	5.77	5.60	5.65	5.62	5.65	5.64	0.063	0.494	0.265	0.417	0.759	0.289
Colour measurements												
L*	55.5	54.2	55.8	52.9	53.5	54.7	1.43	0.213	0.793	0.475	0.330	0.797
a*	6.99	7.55	5.54	6.29	6.12	5.95	0.559	0.218	0.724	0.116	0.056	0.262
b*	4.10	4.39	3.46	3.27	3.25	3.17	0.584	0.121	0.819	0.529	0.392	0.771
TBARS (mg MDA/kg)	0.03	0.03	0.03	0.03	0.02	0.02	0.007	0.112	0.400	0.729	0.618	0.698
Lipid oxidation (mg kg)	7.79	9.95	11.0	11.6	11.0	9.58	1.659	0.406	0.654	0.723	0.925	0.289
WBSF, kg	8.17	8.64	8.19	7.64	8.21	8.22	0.658	0.568	0.436	0.651	0.743	0.907
CL, %	28.8	31.3	26.2	32.4	32.6	33.6	3.05	0.111	0.657	0.822	0.504	0.606

[a,b]Within a row, means with different superscript letters differ, $P < 0.05$.
NPD, normal crude protein diet; RPD, reduced crude protein diet; RPDL, reduced crude protein diet with Leu supplementation.
WBSF, Warner-Bratzler shear force; CL, cooking loss.

4.3.3. Intramuscular fat content and composition

The IMF content and fatty acid composition in *longissimus lumborum* muscle are shown in Table 4.4. Dietary arginine supplementation had no effect neither on IMF content nor fatty acid composition ($P>0.05$). When compared to the pigs fed NPD, those fed RPD and RPDL had greater IMF ($P<0.01$), 16:0 ($P<0.01$), 16:1*c*9 ($P<0.01$), 18:0 ($P<0.05$), 18:1*c*9 ($P<0.01$), SFA ($P<0.01$), MUFA ($P<0.01$) and *n-6* PUFA ($P<0.05$). Furthermore, the percentages of 18:2*n-6* ($P<0.05$) and PUFA ($P<0.05$) increased in pigs fed RPDL when compared with the pigs fed NPD. An interaction between dietary arginine and protein level was observed for *n-3* PUFA content ($P<0.05$).

4.3.4. Free amino acids

Crude protein and free amino acid composition of *longissimus lumborum* muscle of pigs is present in Table 4.5. Content of crude protein in the muscle was not affected by any dietary treatment ($P>0.05$). The glutamine was around 4 µmol/100 g of muscle lower ($P<0.05$) in pigs supplemented with arginine than in their counterparts. Compared with the NPD treatment, the pigs fed RPD had greater alanine ($P<0.05$), histidine ($P<0.001$) and tyrosine ($P<0.05$) and lower arginine ($P<0.001$), glutamine ($P<0.05$) and glycine ($P<0.05$) contents. When compared with pigs fed NPD, pigs fed RPDL had greater histidine ($P<0.01$) and lower values of arginine ($P<0.001$), glutamine ($P=0.001$), glycine ($P<0.05$), isoleucine ($P<0.01$), leucine ($P<0.05$), lysine ($P<0.05$) and valine ($P<0.001$). Differences between RPD and RPDL were found for alanine ($P<0.01$), asparagine ($P<0.05$), isoleucine ($P<0.01$) and valine ($P<0.001$). The contents of these free amino acids in muscle decreased when RPD was supplemented with leucine.

4.3.5. Trained sensory panel analysis

The trained sensory panel scores for *longissimus lumborum* muscle of pigs are presented in Table 4.6. A significant arginine × protein level interaction was detected for tenderness ($P<0.001$), juiciness ($P<0.05$) and overall acceptability ($P<0.05$). Interestingly, within the RPDL, the pork produced with dietary arginine supplementation had greater tenderness and overall acceptability scores than those obtained with the control. Regarding juiciness scores, meat from NPD supplemented with arginine had greater rating than that obtained from NPD in the control treatment. The arginine supplementation had a significant ($P<0.05$) role on pork off-flavour. The meat from arginine supplemented pigs had greater off-flavour values than control pigs (0.96 *vs*. 0.71).

Table 4.4 - Intramuscular fat content (g/100 g muscle) and fatty acid (FA) composition (mg/100 g muscle) of *longissimus lumborum* muscle from pigs.

Item	Control			Arginine			Significance level				
								Dietary protein level			
	NPD	RPD	RPDL	NPD	RPD	RPDL	Arg	NPD *vs*. RPD	NPD *vs*. RPDL	RPD *vs*. RPDL	Arg × Prot
IMF	1.34±0.181	1.85±0.181	2.20±0.181	1.53±0.181	2.30±0.181	2.05±0.181	0.274	0.001	<0.001	0.780	0.261
Major individual FA											
16:0	122±10.2	190±26.4	270±31.0	164±18.7	231±30.2	249±16.6	0.291	0.007	<0.001	0.078	0.302
16:1*c*9	12.3±1.22	22.2±4.05	35.5±5.35	17.8±2.25	29.8±3.77	31.6±2.84	0.292	0.002	<0.001	0.078	0.320
18:0	64.3±5.45	97.5±12.95	132±14.6	85.9±10.91	115±15.3	124±7.96	0.281	0.014	<0.001	0.104	0.374
18:1c9	176±18.1	318±53.7	465±57.7	256±33.9	387±52.9	434±29.8	0.275	0.004	<0.001	0.062	0.337
18:2*n-6*	102±3.46	129±7.66	127±6.65	127±6.16	128±9.79	132±4.75	0.087	0.063	0.012	0.929	0.089
20:4*n-6*	22.5±1.78	30.6±2.02	26.3±1.77	26.1±1.68	25.2±1.90	26.5±1.84	0.717	0.061	0.246	0.431	0.068
Partial sums of FA											
SFA	199±15.7	304±41.2	424±47.7	265±30.9	366±47.5	394±25.5	0.286	0.008	<0.001	0.086	0.321
MUFA	216±21.6	387±64.0	565±70.2	312±39.5	471±62.4	525±35.5	0.281	0.003	<0.001	0.061	0.337
PUFA	142±4.86	181±9.93	174±7.91	174±8.04	172±6.81	178±6.81	0.221	0.051	0.015	0.947	0.050
n-3 PUFA	8.20±0.413^{ab}	9.47±0.537^{b}	8.97±0.380^{b}	9.34±0.476^{b}	7.99±0.618^{a}	7.72±0.340^{a}	0.175	0.937	0.297	0.427	0.012
n-6 PUFA	134±4.51	172±9.53	165±7.58	164±7.63	164±11.5	170±6.49	0.174	0.041	0.009	0.979	0.057

^{a,b}Within a row, means with different superscript letters differ, $P<0.05$.

NPD, normal crude protein diet; RPD, reduced crude protein diet; RPDL, reduced crude protein diet with Leu supplementation.

SFA = 12:0+14:0+15:0+16:0+17:0+18:0+20:0; MUFA = 16:1c7+16:1c9+17:1c9+18:1+18;1c9+18:1c11+20:1c11; PUFA = 18:2*n-6*+18:3*n-3*+20:2*n-6*+20:3*n-6*+20:3*n-3*+20:4*n-6*+20:5*n-3*+22:4*n-6*+22:5*n-3*+22:6*n-3*; *n-3* PUFA = 18:3*n-3*+20:3*n-3*+20:5*n-3*+22:5*n-3*+22:6*n-3*; and *n-6* PUFA = 18:2*n-6*+20:2*n-6*+20:3*n-6*+20:4*n-6*+22:4*n-6*.

Table 4.5 – Crude protein (g/100 g muscle) and free amino acid composition (µmol/100 g muscle) of *longissimus lumborum* muscle from pigs.

	Control			Arginine			SEM	Significance level				
								Arg	Dietary protein level			Arg × Prot
	NPD	RPD	RPDL	NPD	RPD	RPDL			NPD *vs*. RPD	NPD *vs.* RPDL	RPD *vs.* RPDL	
Crude protein	21.2	21.6	21.6	21.7	21.3	21.4	0.310	0.954	0.983	0.914	0.931	0.431
Ala	19.9	22.5	19.5	18.7	22.7	17.5	1.343	0.359	0.017	0.569	0.004	0.709
Arg	3.81	2.59	2.53	3.84	2.93	2.64	0.249	0.339	<0.001	<0.001	0.395	0.728
Asn	0.93	0.99	0.87	0.85	1.06	0.84	0.077	0.812	0.084	0.709	0.037	0.639
Asp	0.12	0.18	0.22	0.15	0.25	0.14	0.048	0.909	0.098	0.350	0.491	0.286
Glu	5.85	5.42	5.09	5.53	5.82	5.53	0.474	0.655	0.875	0.419	0.514	0.668
Gln	32.9	27.6	25.6	28.4	24.3	22.2	1.983	0.026	0.022	0.001	0.310	0.946
Gly	14.9	13.1	14.4	18.4	14.2	13.5	1.317	0.269	0.027	0.046	0.817	0.261
His	1.06	1.46	1.25	1.07	1.52	1.48	0.151	0.254	<0.001	0.007	0.271	0.588
Ile	2.28	2.20	1.68	2.11	2.12	1.62	0.168	0.465	0.856	0.002	0.004	0.942
Leu	4.65	4.43	3.78	4.61	4.67	3.90	0.363	0.717	0.824	0.035	0.057	0.933
Lys	3.63	3.11	3.53	4.82	4.28	3.15	0.612	0.052	0.199	0.035	0.390	0.097
Met	33.9	34.8	32.0	30.6	34.3	31.0	1.675	0.241	0.185	0.638	0.075	0.682
Phe	3.47	3.53	3.12	3.85	3.64	3.19	0.253	0.374	0.757	0.051	0.097	0.797
Pro	3.22	2.65	2.84	3.13	2.86	2.37	0.474	0.759	0.376	0.235	0.758	0.774
Ser	2.99	3.07	2.78	2.99	3.20	2.85	0.210	0.689	0.507	0.387	0.130	0.963
Thr	2.39	2.35	2.18	2.35	2.37	1.85	0.224	0.526	0.947	0.113	0.128	0.699
Trp	2.57	2.74	2.70	2.61	2.74	2.51	0.161	0.713	0.424	0.830	0.558	0.617
Tyr	24.4	29.1	28.5	25.5	27.2	26.3	1.391	0.378	0.025	0.084	0.589	0.425
Val	3.79	3.65	2.69	3.43	3.57	2.52	0.244	0.311	0.994	<0.001	<0.001	0.856

NPD, normal crude protein diet; RPD, reduced crude protein diet; RPDL, reduced crude protein diet with Leu supplementation.; SEM, standard error of mean.

Table 4.6 - Sensory panel scores of *longissimus lumborum* muscle from pigs.

Item	Control			Arginine			SEM	Significance level				
								Arg	Dietary protein level			Arg × Prot
	NPD	RPD	RPDL	NPD	RPD	RPDL			NPD *vs*. RPD	NPD *vs*. RPDL	RPD *vs.* RPDL	
Tenderness	5.08^{ab}	5.31^{bc}	4.74^{a}	5.29^{bc}	5.16^{ab}	5.75^{c}	0.156	0.005	0.742	0.697	0.959	<0.001
Juiciness	3.10^{a}	3.61^{b}	4.12^{c}	3.65^{b}	3.81^{bc}	3.87^{bc}	0.130	0.111	0.011	<0.001	0.025	0.015
Flavour	3.99	4.10	4.39	4.11	4.36	4.23	0.125	0.480	0.166	0.037	0.508	0.212
Off-flavour	0.64	0.61	0.87	0.87	1.16	0.84	0.151	0.037	0.412	0.507	0.856	0.142
Overall acceptability	4.25^{a}	4.44^{ab}	4.22^{a}	4.51^{ab}	4.35^{a}	4.83^{b}	0.147	0.026	0.921	0.309	0.362	0.047

$^{a-c}$Within a row, means with different superscript letters differ, $P<0.05$.
NPD, normal crude protein diet; RPD, reduced crude protein diet; RPDL, reduced crude protein diet with Leu supplementation; SEM, standard error of mean.

4.3.6. Correlation between carcass parameters and meat quality, and between free amino acids and trained sensory panel analysis

The correlation coefficients (r) found among IMF, major fatty acids, ADFI, ADG, G:F, HCW, carcass yield, loin weight, pH_{24}, WBSF, cooking loss and trained sensory panel scores are shown in Table 4.7. In addition, the correlation values between trained sensory panel scores and free amino acids contents are presented in Table 4.8.

As IMF content increases, its composition changed by increasing the proportion of the SFA and MUFA [16:0 (P<0.001), 16:1*c*9 (P<0.001), 18:0 (P<0.01) and 18:1*c*9 fatty acid (P<0.001)] and by decreasing the proportion of the PUFA [18:2*n-6* (P<0.001) and 20:4*n-6* fatty acid (P<0.001)]. In addition, a moderate positive correlation (0.7≥r≥0.3) between IMF deposition and ADFI was also observed (P<0.001). Furthermore, a negative correlation was detected between IMF with carcass yield (P<0.05). However, no significant correlations (P>0.05) were observed between fatty acid contents and carcass yield.

Regarding correlations among growth performance and carcass traits, ADFI and G:F were positively and moderately correlated with ADG (P<0.001). In addition, the HCW was positively and moderately correlated with carcass yield (P<0.001) and loin weight (P<0.001). Greater carcass yield levels were related with increased loin weight (P<0.001) and pH_{24} (P<0.01) values. Regarding trained sensory panel scores, tenderness and juiciness attributes were the significant contributors to pork acceptability. Indeed, high (r>0.70) and moderate correlations were found between acceptability and tenderness (P<0.001) and juiciness (P<0.05), respectively. Juiciness was positively correlated with 16:1*c*9 (P<0.05) and negatively correlated with 18:2*n-6* (P<0.05). A moderate negative correlation (r=-0.42) was found between WBSF and tenderness (P<0.01).

Regarding Pearson's correlations between trained sensory panel scores and free amino acids contents in *longissimus lumborum* muscle (Table 4.8), moderate positive correlations were observed between aspartic acid and juiciness (P<0.01) and off-flavour ratings (P<0.05). Furthermore, a weak positive correlation between histidine and off-flavour (P<0.05) was detected. The increase of tryptophan content in muscle was associated with a decrease of pork tenderness and acceptability scores (P<0.05).

Table 4.7 - Pearson´s correlation coefficients among intramuscular fat content (IMF, g/100 g muscle), major fatty acids (g/100 g total fatty acids), ADFI, (kg/day), ADG (kg/d), G:F (kg/kg), HCW (kg), carcass yield (%), loin weight (kg), pH at 24 h, Warner-Bratzler shear force (WBSF, kg), cooking loss (%) and sensory panel scores of *longissimus lumborum* muscle from pigs.

	16:0	16:1c9	18:0	18:1c9	18:2*n*-6	20:4n-6	IMF	ADFI	ADG	G:F	HCW	Carcass yield	Loin weight	pH_{24}	WBSF	Cooking loss	Tendern	Juicine.	Flavour	Off-flavour
IMF	0.78***	0.77***	0.42**	0.81***	-0.82***	-0.79***														
ADFI	0.51***	0.44**	0.35**	0.35**	-0.40**	-0.43**	0.50***													
ADG	0.30*	0.16	0.29*	-0.02	-0.06	-0.16	0.13	0.62***												
G:F	0.07	-0.06	0.14	-0.25	0.17	0.07	-0.14	0.14	0.86***											
HCW	0.13	0.09	0.07	0.02	-0.01	-0.12	-0.07	-0.04	0.14	0.22										
Carcass yield	-0.04	-0.22	0.12	-0.20	0.21	0.09	-0.28*	-0.25	0.03	0.21	0.58***									
Loin weight	0.02	-0.10	0.08	-0.08	0.09	-0.00	-0.27	-0.17	0.17	0.34*	0.46***	0.52***								
pH_{24}	0.09	-0.16	0.30*	-0.02	0.03	-0.06	-0.12	-0.07	0.04	0.09	0.14	0.41**	0.25							
WBSF	0.08	0.01	-0.02	0.03	-0.02	-0.06	0.16	0.13	0.02	-0.07	0.10	-0.11	0.04	-0.04						
Cooking loss	0.02	-0.09	0.08	-0.01	0.01	-0.03	0.06	0.06	0.16	0.16	0.01	-0.15	0.13	0.18	0.48***					
Tenderness	-0.02	0.00	0.12	0.05	-0.05	-0.01	0.00	-0.12	-0.01	0.08	0.07	0.13	0.11	0.19	-0.42**	0.06				
Juiciness	0.21	0.31*	0.09	0.24	-0.28*	-0.17	0.21	0.14	-0.04	-0.15	-0.03	-0.17	-0.28*	0.03	-0.18	-0.12	0.14			
Flavour	0.24	0.17	0.12	0.10	-0.16	-0.14	0.09	0.06	0.10	0.09	-0.06	0.05	-0.19	0.14	-0.00	0.08	-0.00	0.21		
Off-flavour	0.05	0.08	0.16	0.23	-0.14	-0.22	0.10	0.10	-0.07	-0.16	0.01	-0.06	-0.09	-0.01	-0.32*	-0.25	0.12	0.22	0.14	
Acceptability	0.03	0.06	0.13	0.02	-0.08	0.02	0.03	-0.06	0.03	0.10	0.02	0.15	0.01	0.16	-0.29	0.12	0.84***	0.30*	0.06	-0.15

* $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$.

Table 4.8 - Pearson´s correlation coefficients between free amino acids (µmol/100 g muscle) and sensory panel scores of *longissimus lumborum* muscle from pigs.

	Asp	Glu	Asn	Ser	Gln	His	Gly	Thr	Arg	Ala	Tyr	Val	Met	Trp	Phe	Ile	Leu	Lys	Pro
Tenderness	0.08	0.06	-0.08	0.03	0.01	0.18	-0.13	0.13	-0.17	-0.07	-0.08	-0.15	-0.21	-0.33*	0.04	-0.07	0.15	-0.21	-0.09
Juiciness	0.36**	-0.15	-0.01	0.01	-0.22	0.11	0.05	-0.04	-0.22	0.02	0.21	-0.21	-0.16	0.10	-0.04	-0.18	-0.11	0.21	0.03
Flavour	0.25	0.01	0.05	-0.01	-0.18	0.10	0.01	-0.01	-0.10	-0.03	-0.03	-0.07	0.03	-0.09	-0.06	-0.13	-0.03	-0.00	-0.03
Off-flavour	0.31*	-0.05	0.02	0.06	0.07	0.28*	-0.14	0.05	-0.02	0.07	0.14	0.01	0.09	0.01	0.01	0.02	0.09	0.15	0.07
Acceptability	0.18	0.08	-0.11	-0.03	-0.05	0.09	0.01	0.12	-0.18	-0.05	-0.07	-0.24	-0.25	-0.32*	0.01	-0.16	0.07	-0.21	-0.07

* $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$.

4.4. Discussion

The IMF content influences meat quality and a minimum value of 2.5% has been proposed to achieve a positive effect on eating quality and sensory acceptability of pork (DeVol *et al*., 1988; Fernandez *et al.*, 1999). Therefore, to satisfy consumers, the main objective of the meat industry is to produce pork with increased IMF content and, consequently, to improve meat quality traits. In this study, the IMF content did not increase in pigs fed diets supplemented with arginine. However, some studies with dietary arginine supplementation (*e.g.* Tan *et al*., 2009; Ma *et al.*, 2010) found an increased IMF content without changing pork quality. Contrarily, Go *et al*. (2012) did not find an increased IMF content in pigs fed diets with arginine, which is in agreement with our results. It is well known that arginine regulates the partitioning of dietary energy in favor of muscle protein accretion (Wu *et al*., 2007). Results of recent studies indicate that arginine is involved in important regulatory functions in nutrient metabolism and immune response, thus affecting the efficiency of feed utilization by pigs.

In contrast to dietary arginine supplementation, the reduction of dietary crude protein from 16% in NPD to 13% in RPD and RPDL diets increased the IMF by 45-48%. These results are in agreement with several studies, in which low crude protein diets also increased IMF content in commercial crossbred pigs (*e.g.* 20 *vs*. 16% crude protein, Da Costa *et al*., 2004 and Wood *et al.*, 2004; 21 *vs*. 18% crude protein, Doran *et al*., 2006). Wood *et al*. (2013) reported that a diet with 16% less protein and lower levels of essential amino acids produced fatter pigs with greater IMF contents in the longissimus muscle, when compared with the control diet. Furthermore, it was recently shown by our research group that the increased IMF promoted by RPD in pigs is due to lysine limitation (Madeira *et al.*, 2013a, chapter 2). However, the addition of leucine to reduced protein diet (RPDL) did not result in further improvement of IMF, which is in disagreement with the findings of Hyun *et al*. (2007). The latter reported an increase of IMF content in pigs fed diets with high leucine and low lysine levels.

In the present study, dietary arginine supplementation had no effect on growth performance parameters (ADFI, ADG, G:F), backfat thickness, and loin weight, which is in agreement with some previous studies (*e.g.* Ma *et al*., 2010; Go *et al*., 2012). In contrast, Tan *et al*. (2009) reported that dietary arginine supplementation increases ADG and can beneficially increase protein gain and reduce body fat accretion in pigs. Additionally to the desirable increased IMF, negative effects of low protein diets in pigs were reported, namely slower growth rate, thicker backfat and smaller longissimus muscle area (Goerl *et al*., 1995; Kerr *et al*., 1995). Nonetheless, when the dietary protein level was reduced, with or without leucine addition, the ADG was negatively affected, which is probably explained by lysine reduction (Madeira *et al*.,

2013a, chapter 2). Similar results with diets with leucine addition were found by Hyun *et al*. (2003). However, the ADFI increased in pigs fed RPD but not in pigs fed RPDL, suggesting that leucine supplementation might compensate the lysine deficiency. The ADFI increased in the pigs fed RPD, relative to NPD, because the animals needed to consume more feed to compensate their needs. In addition, RPD and RPDL increased backfat thickness and decreased loin weight.

The IMF and colour influence the appearance of pork at retail (Brewer *et al.*, 2001). The consumer's purchase decision depends on meat colour, which is an important factor of freshness and meat quality (Khliji *et al*., 2010). The pigs fed the diet with leucine addition tended to show a lower a* (P=0.056), the meat was less redness, which can compromise the consumer choice. The arginine supplementation did not affect the colour parameters, in contrast to a previous study, where arginine tended to increase the lightness of muscle at 24 h postmortem, which is consistent with a more rapid pH decline (Go *et al*., 2012). Arginine is a nutritionally important amino acid and plays multiple physiologic functions in animals (Jogben *et al*., 2006; Wu *et al*., 2007). One of these functions is to increase antioxidant ability, reduce superoxide release and attenuate lipid peroxidation (Galli 2007; Petrovic *et al*., 2008). In our study, the arginine or leucine addition did not affect the TBARS and lipid oxidation. However, it is well known that arginine is metabolized to nitric oxide, proline, glutamine and polyamines with enormous biological importance (Liao, Majithia, Huang & Kimmel, 2008).

Tan *et al*. (2011) found that dietary arginine supplementation increase the percentage of oleic acid in skeletal muscle, which may be explained by arginine or nitric oxide dependent activation of stearoyl-CoA desaturase, a key enzyme in the formation of oleic acid (Smith, Tokach, Quinn, Nelssen & Good, 1999). Contrarily to these results, in our study the arginine supplementation did not change oleic acid or MUFA. These results have important implications for meat quality, because pork quality is positively correlated with oleic acid and negatively related with stearic and linoleic acids. Our results showed that protein reduction and leucine addition increased MUFA and decrease PUFA proportions. These results are in accordance to those obtained by Cameron & Enser (1991), who reported that an increase in IMF improves eating quality, increases MUFA proportion and decreases PUFA percentage.

The sensory attributes that contribute the most to a positive acceptability of pork are tenderness, juiciness and flavour (Aaslyng *et al*., 2007). Fernandez *et al.* (1999a) reported that when IMF levels increase above approximately 2.5%, the tenderness, juiciness and flavour are significantly improved. In our study, the IMF levels are below 2.5% and, therefore, the scores of tenderness, juiciness and flavour were low. As consequence, the overall acceptability was also low. Skelley *et al.* (1973) reported that WBSF is a very reliable indicator of meat tenderness. This was confirmed in this study, because the correlation

between meat shear force and tenderness (r=-0.42) was statistically significant (P<0.01). Some studies reported that the contribution of IMF content to meat sensory attributes is small (Wood *et al.*, 1996; van Laack *et al.*, 2001), while others studies reported that IMF has a significant role in sensory parameters (Wood *et al*., 2004; Fortin *et al*., 2005, Cannata *et al*., 2010). However, in our study, IMF did not correlate with any sensory attribute. This is probably due to the low IMF content in all pigs (below 2.5%), which indicates that the increase in IMF observed in pigs fed RPD might not be sufficient to improve pork sensory attributes. Our results showed an increase of juiciness with arginine supplementation in NPD, but IMF did not increase. Moreover, the RPD and RPDL increased juiciness in control pigs, which is in accordance with the increased IMF content in these treatments. The arginine supplementation of NPD resulted in greater values for juiciness than the NPD not supplemented with arginine. The overall acceptability of pork was positively correlated with tenderness and juiciness. Our results also showed an increase in tenderness, and therefore in overall acceptability, in pigs fed reduced protein diets with both arginine and leucine addition.

Although dietary amino acid supplementation could influence meat sensory traits, the studies to investigate these effects are scarce. In the present experiment, dietary arginine supplementation introduced an off-flavour in pork, which was not due to the increment of free arginine in pork. Lorenzo & Franco (2012) reported that arginine, leucine, isoleucine, valine, phenylalanine, methionine and histidine are bitter, glutamic acid and aspartic acid show pleasant fresh taste, and glycine, alanine and serine are sweet. The majority of off-flavours identified by panelists were bitter, sweet, acid, metallic and boar taint. We observed a significant positive correlation between histidine and aspartic acid with general off-flavour scores. This could be an explanation to the off-flavour found in arginine pigs. Also, some arginine derived compounds might be formed in muscle or released during pork thermal processing.

4.5. Conclusions

In conclusion, the results of this study indicate that, in our experimental conditions, dietary arginine supplementation during the growing-finishing phase of commercial crossbred pigs does not affect growth performance, IMF and pork quality traits, but slightly affected some sensory attributes. In addition, our data confirm that low crude protein diets increase IMF content in lean pig genotypes but decrease animal performance. However, dietary leucine supplementation of low crude protein diets does not seem to have any additional effect on IMF or meat quality traits. Moreover, the data indicate that the cumulative effect of a low crude protein diet, leucine and arginine does not increase IMF, relative to the individual effect

of the low crude protein diet, but results in an increased pork tenderness and overall acceptability. However, the small increment in pork eating traits obtained with this combined feeding strategy indicates that this dietary approach may have limited use for the meat industry and consumers.

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Chapter 5

COMBINED EFFECTS OF DIETARY ARGININE, LEUCINE AND PROTEIN LEVEL ON FATTY ACID COMPOSITION AND GENE EXPRESSION IN MUSCLE AND SUBCUTANEOUS ADIPOSE TISSUE OF CROSSBRED PIGS

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Contribution of Marta S. Madeira to this article:

Marta S. Madeira collaborated in the animal experiment, participated in the tissue sampling, did the IMF and fatty acids analysis, data processing and statistical analysis. In addition, Marta S. Madeira collaborated in the gene expression analysis, interpretation of the results and in the writing of the manuscript.

Combined effects of dietary arginine, leucine and protein level on fatty acid composition and gene expression in muscle and subcutaneous adipose tissue of crossbred pigs

Abstract

The cumulative effects of dietary arginine, leucine and protein levels on fat content, fatty acid composition and mRNA level of genes controlling lipid metabolism in pig *longissimus lumborum* muscle and subcutaneous adipose tissue (SAT) were investigated. The experiment was performed on fifty four intact male pigs (Duroc × Pietrain × Large White × Landrace crossbred), from 59 to 92 kg of live weight. Pigs were randomly assigned to one of six experimental treatments (*n*=9). The treatments followed a 2 × 3 factorial arrangement, with two levels of arginine supplementation (0 vs. 1%) and three levels of basal diet (normal protein diet, NPD; reduced protein diet, RPD; and RPD with leucine addition to 2.0%, RPDL). The results showed that dietary arginine supplementation did not affect intramuscular fat (IMF) content and backfat thickness but increased total fat in SAT. This effect was associated with an increase of *FASN* and *SCD* mRNA levels in SAT, which suggest that arginine might be involved in the differential regulation of some key lipogenic genes in pig muscle and SAT. The increase in IMF content under the RPD, with or without leucine supplementation, was accompanied by increased *FASN* and *SCD* mRNA levels. Arginine supplementation did not influence the percentage of main fatty acids, whilst RPD had a significant effect on fatty acid composition in both tissues. Leucine supplementation of RPD did not change IMF, total fat of SAT and backfat thickness, but increased 16:0 and 18:1*c*9 and decreased 18:2*n-6* in muscle.

Keywords: pig, arginine, leucine, reduced protein diet, intramuscular fat, fatty acid composition, lipid metabolism

5.1. Introduction

Pork is the most consumed meat in EU countries, with 22,387,604 tons of carcass produced in 2011 (Eurostat, 2013). The genetic selection of commercial pig lines has reduced subcutaneous fat content with the concomitant decreasing of marbling or IMF. The carcass leanness varies between breeds and is generally higher in white European breeds (Large White and Landrace) when compared with Duroc crossbreds (Wood *et al.*, 2004). IMF content is one of the key meat quality traits, and the sensory properties of pork are negatively affected when IMF is reduced below 2-2.5% (De Vol *et al.*, 1988). According to Daszkiewicz *et al.* (2005), about 84% of the carcasses from commercial pig genotypes have a *longissimus lumborum* muscle fat content below the level required for acceptable eating quality. Furthermore, not only fat content but also fatty acid composition of IMF plays an important role in meat quality, and an appropriate ratio of SFA, MUFA and PUFA should be maintained in order to assure superior eating quality and nutritional value of meat (Wood *et al.*, 2008). Therefore, production of pork with high amounts of IMF and a balanced fatty acid composition, without an increase in subcutaneous fat (improved fat partitioning), is one of the main pig industry goals.

Some feeding strategies have been suggested to improve fat partitioning in pigs, mainly based on dietary amino acid supplementation and RPDs. Arginine is a semi-essential amino acid that, in addition to playing multiple physiological functions in animals, enhances lipolysis through the expression of key genes responsible for activation of fatty acid oxidation, in a tissue-specific manner (Jobgen *et al*., 2006; Tan *et al.*, 2011). Previous research on growing finishing pigs suggested that dietary supplementation with arginine increases IMF, thus improving fat partitioning (Tan *et al.*, 2009). This was thought to be due to arginine-induced modulation of lipogenesis and lipolysis regulation in muscle (lipogenesis increased) and white adipose tissue (lipolysis increased) (Tan *et al.*, 2011). Furthermore, the essential amino acid leucine is known to play an important role in control of protein synthesis and insulin release. It is the only amino acid in pigs that can be used exclusively for fat synthesis (ketogenic), and may be converted to acetyl-CoA for fatty acid synthesis in muscle tissue (Hyun *et al.*, 2007). Some studies suggested that IMF of pork can be increased by feeding to finishing pigs with high levels of leucine (Hyun *et al.*, 2003) or high levels of leucine in combination with low levels of lysine (Hyun *et al.*, 2007).

In addition to the supplementation of pig diets with arginine and leucine, the use of RPDs for increasing IMF content in pigs, with less effect on subcutaneous fat deposition, have also been described (Doran *et al.*, 2006). The mechanisms of tissue-specific effects of RPD are not clear (Wood *et al.*, 2008). One of the possibilities is a dietary-stimulated increase in SCD activity in pig muscle, but not in SAT (Doran *et al*., 2006). Our recent data suggested the

existence of breed- and tissue-specific mechanisms of fat deposition in pigs (Madeira *et al.*, 2013b, chapter 3). The results indicated that the RPD increased IMF content in lean pig genotypes but not in fat ones, and the effects of RPD on lipogenesis are tissue-specific mediated via up-regulation of lipogenic enzymes in muscle and adipose tissue.

In pigs, the white adipose tissue is the main site for *de novo* fatty acid biosynthesis and lipogenesis. In contrast, muscle is one of the tissues playing the main role in metabolism of glucose and degradation of lipids (Bergen & Mersmann, 2005). It is known that the regulation of adipogenesis and lipogenesis is a complex process, and a range of the transcription factors play the key role in this regulation. This transcription factors include SREBP1, CEBPα and PPARγ (Zhao *et al.*, 2010). Furthermore, MLX interacting protein-like (MLXIPL) is a critical glucose-responsive transcription factor that regulates lipogenic and glycolitic genes, highly controlled by the insulin-regulated glucose transporter GLUT4 in adipose tissue (Herman *et al.*, 2012). Moreover, the MLXIPL also regulates various enzymes involved in glycolysis and lipogenesis, such as ACACA and FASN (Dentin *et al.*, 2004). ACACA (Liu *et al.*, 1994) and FASN (Clarke, 1993) are the key lipogenic enzymes controlling the rates of SFA biosynthesis, and SCD catalyses the rate-limiting step of MUFA biosynthesis. FADS1, encoding for Δ5 desaturase, and FADS2, encoding for Δ6 desaturase, are membrane-bound enzymes that catalyse the synthesis of PUFA (Nakamura & Nara, 2004). Furthermore, carnitine palmitoyltransferase 1 (CPT-1B) and CRAT are rate-limiting enzymes of lipid catabolism responsible for the transport of fatty acid esters from cytosol to mitochondria for β-oxidation (Zhao *et al.*, 2010). LPL is the rate-limiting enzyme for the conversion of chylomicrons and VLDL into chylomicron remnants and LDL in tissues. Therefore, LPL controls triacylglycerols partitioning between adipose tissue and muscle, thereby increasing fattening or providing energy in the form of fatty acids for muscle growth (Hocquette *et al.*, 1998). Finally, FABP4 is the responsible for fatty acids transport in the adipocytes (Hocquette *et al.*, 2010). However, in spite of a large amount of information regarding the links between gene expression, lipogenic enzymes activity and fat partitioning in pigs in general, it is remains unclear whether and how these processes contribute to dietary regulation of fat partitioning in pigs.

Recent results from our research group strongly indicated that adipogenesis and lipogenesis are regulated differently in the muscle and SAT of commercial crossbred pigs (Madeira *et al*., 2013b, chapter 3). In addition, it was shown that the increased IMF promoted by RPD is very likely due to lysine limitation and it is mediated via up-regulation of the adipogenic transcription factor PPARγ and the lipogenic enzyme SCD. Taking into account the mentioned above, the aim of this study was: (i) to explore the cumulative effects of dietary arginine supplementation, RPD and RPD with leucine supplementation on fat partitioning and fatty acid composition in commercial crossbred pigs; and (ii) to investigate

whether the effects of arginine and leucine are mediated via tissue-specific expression of genes controlling lipid metabolism.

5.2. Material and Methods

5.2.1. Animals and diets

This trial was conducted at the facilities of UIPA-INIAV, and all the experimental procedures involving animals were reviewed by the Ethics Commission of the (CIISA/FMV) and approved by the Animal Care Committee of the National Veterinary Authority (Direcção-Geral de Veterinária), following the appropriated European Union guidelines (Directive 86/609/EEC). All the members of staff involved in the animal experiments hold licence for conducting experiments on live animals from the Portuguese Veterinary Services. Fifty four commercial crossbred (25% Duroc, 25% Pietrain, 25% Large White, and 25% Landrace) entire male pigs with an initial body weight of 58.9 ± 1.59kg were used. Animals were fed a standard concentrate diet from weaning until the beginning of the experiment. All the animals were randomly assigned to one of the six diets in a 2 × 3 factorial arrangement (arginine treatment or an isonitrogenous control, and two protein levels with or without leucine addition).

The diets were isoenergetically formulated (14 MJ ME/kg) and differed in crude protein, arginine and leucine contents, as follows: 16.0% of crude protein (normal protein diet, NPD); 13.0% of crude protein (reduced protein diet, RPD); and 13.0% of crude protein plus L-leucine in the diet to achieve 2% (reduced protein diet with leucine, RPDL). The arginine treatment and the isonitrogenous control were obtained through supplementation of the basal diets with 1.0% of L-arginine and 2.05% of L-alanine, respectively. Arginine or alanine was added to the basal diet at the expense of corn starch to obtain isoenergetic diets. The amino acids were obtained from Fh Diedrichs & Ludwig Post (Mannheim, Germany). The ingredients, chemical composition and fatty acid profile of the experimental diets are shown in Table 5.1. The animals were housed in three pens of three pigs per each treatment (n=9). During the experiment, the animals were fed individually twice a day and had access to water *ad libitum*. Feed offered and refusals were recorded daily in order to calculate feed intake. Pigs were weighed weekly, just before feeding, throughout the experiment.

Table 5.1 - Ingredients, chemical, amino acids and fatty acid compositions of the experimental diets.

	Control			Arginine		
Diets	NPD	RPD	RPDL	NPD	RPD	RPDL
Ingredients (%)						
Maize	62.9	67.3	75.0	63.7	72.3	74.5
Barley	10.0	15.0	8.00	10.0	10.0	10.0
Soybean meal	18.9	10.9	9.60	16.3	7.80	7.2
Sunflower meal	1.64	0.44	-	4.56	4.66	1.98
Soybean oil	1.15	0.98	0.99	1.06	0.88	0.85
Calcium carbonate	0.73	0.73	0.71	0.72	0.70	0.71
Bi-calcium phosphate	1.21	1.32	1.38	1.22	1.35	1.39
Sodium bicarbonate	0.11	0.01	-	0.14	0.06	0.07
Salt	0.35	0.43	0.44	0.33	0.39	0.38
L-Lys	0.30	0.12	0.17	0.34	0.17	0.21
L-Met	0.06	-	-	0.06	-	-
L-Thr	0.07	-	-	0.08	-	-
L-Ala	2.05	2.05	2.05	-	-	-
L-Arg	-	-	-	1.00	1.00	1.00
L-Leu	-	0.17	1.14	-	0.17	1.17
Vitamin-trace mineral premix[1]	0.40	0.40	0.40	0.40	0.40	0.40
Acid mixture[2]	0.10	0.10	0.10	0.10	0.10	0.10
Antioxidant mixture[3]	0.005	0.005	0.005	0.005	0.005	0.005
Chemical composition (% diet)						
DM	87.5	87.7	87.8	87.7	87.7	87.9
Crude protein	16.0	13.1	13.1	15.9	12.9	12.7
Starch	38.3	42.6	42.5	38.5	42.5	43.1
Crude fat	3.36	3.46	3.54	3.46	3.46	3.56
Crude fibre	4.38	3.22	3.06	4.66	4.20	3.36
Ash	3.88	3.78	3.78	4.16	3.98	3.80
Calcium	0.66	0.73	0.75	0.59	0.68	0.71
Phosphorus	0.49	0.51	0.52	0.51	0.52	0.52
ME (MJ ME/kg)	13.8	14.1	14.3	13.9	14.1	14.3
Amino acid composition (% diet)						
Ala	3.13	3.25	3.52	0.16	0.51	0.33
Arg	1.05	0.83	0.49	1.84	1.60	1.56
Asp	0.49	0.35	0.31	0.45	0.38	0.30
Glu	2.07	1.54	1.38	1.82	1.59	1.34
Gly	0.43	0.35	0.41	0.63	0.43	0.41
His	2.02	1.21	0.92	1.27	1.02	0.90
Ile	0.45	0.32	0.38	0.50	0.35	0.35
Leu	1.01	0.93	1.51	0.95	0.94	1.74
Lys	0.84	0.47	0.45	0.70	0.43	0.43
Met	0.02	0.04	0.07	0.06	0.18	0.10
Phe	0.68	0.47	0.28	0.39	0.33	0.31
Pro	0.83	0.79	0.61	0.85	0.96	0.89
Ser	0.81	0.67	0.61	0.78	0.63	0.57
Thr	0.17	0.10	0.12	0.20	0.19	0.18
Tyr	0.31	0.20	0.18	0.24	0.17	0.13
Val	0.70	0.56	0.44	0.57	0.47	0.45
Fatty acid composition (% total fatty acids)						
16:0	15.0	15.3	14.9	15.0	15.0	14.9
18:0	2.72	2.47	2.65	2.58	2.43	2.38
18:1*c*9	24.9	25.0	25.8	24.9	25.4	25.6
18:1*c*11	1.05	0.97	0.98	1.01	0.95	0.94
18:2*n-6*	53.0	53.1	52.8	53.2	53.4	53.3

18:3*n-3*	3.32	3.10	2.85	3.22	2.77	2.77

Dietary treatments: NPD, normal crude protein diet; RPD, reduced crude protein diet; RPDL, reduced crude protein diet with Leu supplementation; ME, metabolisable energy.
[1]Vita Tec (Tecadi, Santarém, Portugal). Provided per kilogram of diet: vitamin A, 6,000 IU; vitamin D3, 1,500 IU; vitamin E (acetate dl-alpha-tocopherol), 15 mg; vitamin B2, 0.3 mg; vitamin B12, 3.75 mg; biotin, 0.1 mg; calcium pantothenate, 12 mg; nicotinic acid, 15 mg; folic acid, 0.75 mg; choline chloride, 200 mg; Cu (cupric sulfate pentahydrate), 15 mg; Zn (zinc oxide), 100 mg; Mn (manganese oxide), 35 mg; I (potassium iodide), 0.7 mg; Co (basic cobaltous carbonate mono hydrous), 0.05 mg; Se (sodium selenite), 0.2 mg; Fe (ferrous carbonate), 80 mg; and BHT, 0.2 mg.
[2]Formic acid, propionic acid, citric acid, and calcium salts (Ultracid V Dry EU; Tecadi).
[3]Ethoxyquin, propyl gallate, and citric acid (Oxi-Nil Dry Premix; Tecadi).

5.2.2. Slaughter and sampling

Feed was removed 17-19 h before animals' slaughter. Pigs were slaughtered at an average live body weight of 91.7 ± 1.61 kg, with no significant differences (P>0.05) between animal groups, at the UIPA Experimental Abattoir (INIAV). Immediately after electrical stunning and exsanguination, samples of *longissimus lumborum* muscle and SAT were collected from the right side of carcass at the first lumbar vertebra level for gene expression analysis. The samples were rinsed with sterile RNAse-free cold saline solution, cut into small pieces (thickness of approximately 0.3 cm), stabilised in RNA Later solution (Qiagen) and stored at -80 ºC until analysed. For analysis of IMF and fatty acid composition, *longissimus lumborum* muscle and SAT samples were collected after slaughter from the right carcass side between third and fifth lumbar vertebras. Muscle was collected and trimmed of visible connective and adipose tissues before blended in a food processor. The samples of muscle and SAT were vacuum packed and stored at -20 ºC until analysed. Backfat thickness was measured in left carcass side at P_2 (last rib position) location.

5.2.3. Feed analysis

Feed samples, collected five times during the trial (the first collection was in the beginning of the trial, followed by regular collections with a 3-weeks interval until the slaughter), were analysed for DM by drying a sample at 100 ºC to a constant weight. Nitrogen content was determined by Kjeldahl method (AOAC, 2000), and crude protein was calculated as 6.25 × N. Crude fibre was determined by the procedure described by the AOAC (2000). The samples were extracted with petroleum ether, using an automatic Soxhlet extractor (Gerhardt Analytical Systems, Königswinter, Germany), to determine crude fat. Analysis of ash and starch contents was carried out according to the procedures described by the AOAC (2000) and Clegg *et al.* (1996), respectively. Gross energy in the feed was determined by adiabatic bomb calorimetry (Parr 1261, Parr Instrument Company). Fatty acid methyl esters (FAME) of the feed samples were analysed by one-step extraction and transesterification, using heptadecaenoic acid (17:0) as internal standard (Sukhija & Palmquist, 1998). Total amino acids were extracted from feed according to the method described by the AOAC (2005). The

extract was analysed by HPLC (Agilent 1100, Agilent Technologies, Avondale, PA, USA) to quantify total amino acids in the feed, according to the procedure described by Henderson *et al.* (2000).

5.2.4. Intramuscular fat and fatty acid composition

The *longissimus lumborum* muscle and SAT samples were lyophilised (-60 °C and 2.0 hPa) to constant weight using a lyophilisator (Edwards High Vacuum International, West Sussex, UK), kept dry at -20 °C and analysed within two weeks. The total fat content of muscle samples (IMF) and SAT was determined using fresh samples by hydrolysis with 4 M HCl followed by Soxhlet extraction for 6 h with petroleum ether (AOAC, 2000). For fatty acid analysis of *longissimus lumborum* muscle and SAT samples, FAME were extracted from the lyophilised samples (approximately 250 and 50 mg, respectively), according to the Folch & Stanley (1957) method, using dichloromethane and methanol (2:1 v/v) instead of chloroform and methanol (2:1 v/v), as described by Carlson (1985). All the extraction solvents contained 0.01% butylated hydroxytoluene as an antioxidant. Fatty acids were converted to methyl esters by a combined transesterification procedure with NaOH in anhydrous methanol (0.5 M), followed by HCl:methanol (1:1 v/v), at 50 °C during 30 and 10 min, respectively, according to Raes *et al.* (2001).

Quantification of FAME in muscle and SAT was performed using a gas chromatograph HP7890A (Hewlett-Packard), equipped with a flame ionization detector (GC-FID) and a Supelcowax^{TM} 10 capillary column (30 m × 0.20 mm i.d., 0.20 µm film thickness; Supelco, Bellefonte, CA). The column temperature of 150 ºC was held for 11 min, then increased to 210 ºC at a rate of 3 ºC/min, and maintained for 30 min. Helium was used as carrier gas at a flow rate of 1.3 mL/min, the split ratio was 1:20 and 1 µl of sample was injected. The injector and detector temperatures were 250 ºC and 280 ºC, respectively. The quantification of total FAME was done using nonadecanoic acid (19:0) as the internal standard. Results for each fatty acid were expressed as a percentage of the sum of detected fatty acids (% total fatty acids).

5.2.5. RNA isolation and complementary DNA synthesis

Total RNA from SAT samples was isolated using RNeasy lipid tissue mini kit (Qiagen). For the *longissimus lumborum* muscle samples, total RNA was isolated with TRIzol Reagent (Invitrogen Gaithersburg, MD, USA) and purified with RNeasy Mini Kit (Qiagen). All the procedures were performed in accordance to the manufacturer's protocols, and all RNAs were subjected to an on-column DNAse I (Qiagen) treatment to remove any contaminating genomic DNA. RNA concentration was determined by analysis of absorbance at 260 nm

using a NanoDrop ND-2000c spectrophotometer (Nanodrop, Thermo Fisher Scientific, Willmington, DE, USA). The A260/280 ratios were between 1.9 and 2.1, and RNA integrity was evaluated using electrophoresis with 1.5% agarose and ethidium bromide staining (1.25 ng/µl, Sigma-Aldrich, St Louis, MO, USA).

One microgram of total RNA was reversed transcribed using with the High Capacity cDNA Reverse Transcription Kit, based on the use of both oligodT and random hexamers as primers, following the manufacturer's protocol (Applied Biosystems) and as was previously described (Madeira *et al*., 2013b, Chapter 3). Control reactions were carried out in the absence of reverse transcriptase in order to check for DNA contamination. cDNA quality was tested by end-point PCR, amplifying all housekeeping and target genes used in this study. The obtained cDNA was divided in aliquots and stored at -20 ºC until further analysis.

5.2.6. Real-time quantitative PCR

Genes used in this study were selected based on their role in the transcriptional control of adipogenesis regulation/differentiation (*MLXIPL*, *PPARγ*, *SREBF1*, *CEBPα*), regulation of lipogenesis (*ACACA*, *FASN*, *FADS1*, *FADS2*, *SCD*), glucose uptake (*GLUT4*), fatty acid uptake (*LPL*) and lipid oxidation (*CRAT*, *CPT1*, *PPARα*) (Table 5.2). Gene specific intron-spanning primers were designed with the aid of Primer3 (http://frodo/wi.mit.edu/primer3/) and Primer Express 2.0 software (Applied Biosystems), based on *Sus scrofa* sequences (www.ncbi.nlm.nih.gov), to generate amplicons ranging in size from 71 to 145 bp. Sequence of primers, GenBank accession numbers, amplicon length and span exons for PCR products is provided in Table 5.2. Primers were synthesized commercially by NZYTech (Lisbon, Portugal). Sequence homology searches against the database of GenBank showed that these primers were specific to the sequence to which they were designed. In order to test the primers and verify the amplified products, a conventional PCR was carried out for all genes investigated in this study before performing the real-time quantitative PCR experiments. Briefly, genes were amplified by conventional qualitative PCR (using 1 µl of cDNA) using the same primers designed for real-time PCR. PCR products were extracted from gels using QIAquick® Gel Extraction Kits (Qiagen). The fragments were then cloned into the pGEM®-Teasy cloning vector (Promega), transformed into pMOS Blue *Escherichia coli* and selected on LB-agar plates containing ampicillin (50 µg/mL). Plasmids containing inserts of right size were sequenced by Stabvida (Portugal) and homology searches were performed with Blast (www.ncbi.nlm.nih.gov/blast) to confirm the identity of amplified fragments. The PCR efficiency was calculated for each amplicon, in triplicate, using the StepOnePlus PCR System software (Applied Biosystems), by amplifying 5-fold serial dilutions of pooled cDNA. All primer sets exhibited an efficiency ranged between 90 and 110%, and the correlation coefficients were higher than 0.99.

Table 5.2 - Characterisation of the selected genes used in the real-time quantitative PCR assay.

Gene symbol	Full gene name	GenBank accession number	Forward primer	Reverse primer	Product size (bp)	Spanned Coding exons
ACACA	Acetyl-coenzyme A carboxylase alpha	NM_001114269	ggccatcaaggacttcaacc	acgatgtaagcgccgaactt	120	46-47
CEBPα	CCAAT/enhancer binding protein (C/EBP), alpha	XM_003127015	ggccagcacacacacattaga	cccccaaagaagagaaccaag	71	1
CPT1B	CPT1B carnitine palmitoyltransferase 1B	NM_001007191.1	cagatggagcggatgttcaa	gagccctcgtagagccacac	133	8-9
CRAT	Carnitine acetyltransferase	NM_001113047	ggcccaccgagcctacac	atggcgatggcgtaggag	138	12-13
FABP4	Fatty acid binding protein 4, adipocyte	NM_001002817	gggccaggaatttgatgaag	ctttccatcccacttctgcac	103	2-3
FASN	Fatty acid synthase	NM_001099930	acaccttcgtgctggcctac	atgtcggtgaactgctgcac	112	40-41
FADS1	Fatty acid desaturase 1	NM_001113041.1	ccactgttggggctgaagg	gatgtgcatggggatgtggt	108	8-9
FADS2	Fatty acid desaturase 2	NM_001171750.1	gccttacaaccaccagcatga	aggccaagtccacccagtc	122	6-7
GLUT4	SLC2A4 solute carrier family 2, member 4	NM_001128433	gctgcctcctacgagatgct	tggccagctggttgagtgt	145	4-5
LPL	Lipoprotein lipase	NM_214286	atctgcgggatacaccaagc	ccaaggctgtatcccaggag	110	3-4
MLXIPL	MLX interacting protein-like	XM_003481002	tgacatgatccagcctgacc	gggggctcagagaagtttga	126	7-8
PPARα	Peroxisome proliferator-activated receptor alpha	NM_001044526	tttccctctttgtggctgct	ggggtggttggtctgcaag	128	5-6
PPARγ	Peroxisome proliferator-activated receptor gamma	NM_214379	gagggcgatcttgacaggaa	gccacctctttgctctgctc	124	6-7
SCD	Stearoyl-CoA desaturase (delta-9-desaturase)	NM_213781	agccgagaagctggtgatgt	gaagaaaggtggcgacgaac	140	5-6
SREBP1	sterol regulatory element binding transcription factor 1	NM_214157	gtgctggcggaggtctatgt	aggaagaagcgggtcagaaag	96	11-12
HouseKeeping genes						
RPLP0	Ribosomal phosphoprotein large, P0 subunit	NM_001098598	tccaggctttaggcatcacc	ggctcccactttgtctccag	95	4-5
RSP29	Ribosomal protein S29	NM_001001633	ggtcagggttctcgctcttg	cactggcggcacatattgag	120	1-2
RPL27	Ribosomal protein L27	NM_001097479.1	gtactccgtggatatccccttg	aacttgaccttggcctctcga	102	1

The gene expression profile of five candidate references genes (glyceraldehyde-3-phosphate dehydrogenase, *GAPDH*, 60S ribosomal protein L27, *RPL27*, ornithine decarboxylase antizyme 1, *OAZ1*, ribosomal protein large P0, *RPLP0,* and 40S ribosomal protein S29, *RPS29*) were analysed in twenty four randomly selected different samples (four pigs from each group). The geNorm algorithm (Vandesompele *et al*., 2002) and NormFinder algorithm (Andersen *et al*., 2004) were used to evaluated their stability in all the samples. *RPLP0* and *RPS29* were identified as the most stable pair of endogenous controls genes for normalisation of results in *longissimus lumborum* muscle, whereas *RPLP0* and *RPL27* genes were identified as the most stable pair for SAT. Quantitative RT-PCR (qRT-PCR) reactions were carried out using MicroAmp Optical 96-well plates (Applied Biosystems) in a StepOnePlus thermocycler (Applied Biosystems) in standard cycling conditions. Measurements of each sample for each gene were conducted in duplicate. 12.5 µl of PCR reaction mixtures contained 6.25 µl of 2×Power SYBR Green PCR Master Mix (Applied Biosystems), 160 nM of gene-specific forward and reverse primers, and 2 µl of diluted cDNA as a template. No transcription and no template samples were used as controls. A melting curve analysis was performed after the final cycle to ensure specificity of primer and absence of primer dimer formation. The relative amount of each target gene was calculated using the geometric mean of *RPLP0/RPS29* and *RPLP0/RPL27* as a normaliser for muscle and SAT, respectively. The relative gene expression levels were calculated using the Livak & Schmittgen (2001) method, corrected for variation in amplification efficiency, as described by Fleige *et al.* (2006).

5.2.7. Statistical analysis

All data were checked for normal distribution and variance homogeneity. As variance heterogeneity was detected for most fatty acids and genes, these data were analysed using Proc MIXED of the SAS software package (version 9.2; SAS Institute), with a model including the dietary arginine and protein level with or without leucine supplementation and their respective interaction, as fixed effects, and the repeated statement considering the group option for accommodate the variance heterogeneity. As IMF, total fat of SAT and P_2 had variance homogeneity, these data were analysed using the same model, but without the repeated statement. If the interaction between dietary arginine and protein level was significant, multiple comparisons of least square means were determined using the PDIFF with Tukey-Kramer adjustment options of SAS. The contrasts between dietary protein level (NPD *vs*. RPD, NPD *vs.* RPDL and RPD *vs.* RPDL) were performed. The level of significance was set at $P<0.05$.

The need for covariate adjustment was explored using animal age, live and slaughter weights, IMF, total fat (SAT) and P_2 as covariates, but only IMF and total fat were found to be

significant for several variables. Thus, IMF and total fat were retained as covariates for some muscle and SAT variables, respectively. For each variable, where the use of covariate was justified, the structure of covariate model was determined according to procedures described by Milliken & Johnson (2002) and ranged to simple slope model to individual slopes for each arginine × protein level combinations. The adjusted variables and their covariance models are identified in tables' footnote.

Pearson correlation matrices were computed using the PROC CORR of SAS. When needed, adjusted variables to the common mean IMF in muscle and the common mean total fat in SAT were used to compute Pearson correlations.

5.3. Results

This chapter discusses the results of the present trial in commercial pigs regarding fat content and fatty acid composition in *longissimus lumborum* muscle and SAT. Furthermore, the possible molecular mechanisms underlying fat deposition in muscle and SAT were elucidated through the assessment of mRNA expression level of genes encoding for key lipogenic enzymes and transcription factors.

5.3.1. Intramuscular fat and fatty acid composition of muscle

Results of IMF, fatty acid composition and partial sums of fatty acids in the *longissimus lumborum* muscle are presented in Table 5.3. IMF content was not affected neither by dietary arginine (P=0.274) nor by leucine supplementation of RPD (P=0.780). However, the reduction of protein level in the diet resulted in a significant (P≤0.001) increase in IMF content by 45% and 48% for the RPD and RPDL groups, respectively.

The predominant fatty acids in IMF were 18:1*c*9 (32-37% of total FAME), 16:0 (21-22%), 18:2*n-6* (12-16%), 18:0 (11-12%), 18:1*c*11 (4%) and 20:4*n-6* (3-4%) for all experimental groups. The term "others" in the Table 5.3, refers to unidentified minor fatty acids and the dimethylacetals 16:0, 18:0 and 18:1, which are derived from plasmalogens. Arginine treatment affected only the percentage of 3 of the 24 fatty acids identified in the muscle (12:0, 18:1*c*11 and 22:5*n-3*). The dietary protein or leucine levels affected the proportion of 19 individual fatty acids in the muscle. The proportion of 12:0, 16:1*c*7 and 18:3*n-3* was lower in the pigs fed RPD and RPDL when compared to the animals fed NPD. Contrarily, 18:1*c*11 was higher in the RPD and RPDL than in the NPD. The RPDL increased the proportion of 18:1*c*9 and 20:1*c*11 and decreased the proportion of 18:2*n-6* when compared to the NPD and RPD. The pigs fed RPDL had lower proportions of 20:3*n-6* (P=0.008), 20:4*n-6* (P=0.006), 20:5*n-3* (P=0.006) and 22:5*n-3* (P=0.031) than pigs fed RPD.

Table 5.3 - Effect of dietary arginine, leucine and protein levels on intramuscular fat (IMF; % muscle), fatty acid composition (% total fatty acids), partial sums of fatty acids and related ratios in the *longissimus lumborum* muscle of pigs.

	Control						Arginine						Significance level				
	NPD		RPD		RPDL		NPD		RPD		RPDL		Arg	Dietary protein level			Arg×Prot
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE		NPD *vs.* RPD	NPD *vs.* RPDL	RPD *vs.* RPDL	
IMF	1.34	0.181	1.85	0.181	2.20	0.181	1.53	0.181	2.30	0.181	2.05	0.181	0.274	0.001	<0.001	0.780	0.261
Fatty acid composition																	
12:0*	0.25	0.019	0.19	0.017	0.17	0.013	0.20	0.012	0.19	0.008	0.16	0.004	0.027	0.039	<0.001	0.004	0.185
14:0*	0.99	0.033	0.92	0.031	1.04	0.041	1.01	0.041	1.01	0.036	1.04	0.022	0.201	0.409	0.254	0.025	0.518
15:0†	0.21	0.022	0.20	0.011	0.16	0.012	0.17	0.020	0.19	0.010	0.14	0.006	0.088	0.094	0.076	0.103	0.223
16:0*	21.6	0.38	20.6	0.28	21.6	0.32	21.3	0.28	21.1	0.29	21.5	0.22	0.952	0.051	0.858	0.011	0.372
16:1*c*7*	0.32	0.011	0.29	0.011	0.26	0.012	0.34	0.015	0.28	0.004	0.30	0.012	0.060	<0.001	<0.001	0.566	0.068
16:1*c*9*	2.36	0.121	2.35	0.093	2.66	0.109	2.41	0.080	2.61	0.129	2.67	0.125	0.237	0.427	0.025	0.109	0.509
17:0*	0.40	0.046	0.39	0.026	0.35	0.033	0.43	0.048	0.44	0.028	0.33	0.025	0.573	0.932	0.097	0.010	0.471
17:1*c*9	0.26	0.025	0.21	0.017	0.21	0.025	0.21	0.016	0.21	0.016	0.22	0.015	0.418	0.161	0.316	0.758	0.397
18:0‡	11.8	0.25	10.6	0.26	10.7	0.19	11.4	0.20	10.7	0.27	10.8	0.20	0.636	0.047	0.093	0.739	0.254
18:1*	0.12	0.008	0.11	0.007	0.12	0.006	0.11	0.004	0.13	0.008	0.13	0.004	0.636	0.320	0.040	0.328	0.191
18:1*c*9*	32.3	1.35	33.7	0.81	36.1	1.15	33.9	0.71	34.2	0.88	37.1	0.53	0.190	0.389	0.002	0.004	0.848
18:1*c*11§	3.79	0.102	3.87	0.072	3.98	0.060	3.59	0.076	4.01	0.084	4.02	0.094	0.028	0.007	0.001	0.406	0.162
18:2*n-6**	15.7	1.00	15.0	0.63	12.7	0.82	15.5	0.62	14.5	0.61	12.5	0.30	0.594	0.307	<0.001	<0.001	0.961
18:3*n-3*	0.41	0.022	0.35	0.015	0.29	0.023	0.44	0.023	0.34	0.018	0.30	0.006	0.780	<0.001	<0.001	0.006	0.977
20:0*	0.16	0.012	0.13	0.008	0.15	0.009	0.13	0.007	0.13	0.009	0.14	0.008	0.089	0.166	0.687	0.258	0.268
20:1*c*11*	0.57	0.024	0.56	0.023	0.64	0.024	0.56	0.031	0.51	0.018	0.62	0.022	0.211	0.221	0.032	<0.001	0.647
20:2*n-6**	0.42	0.016	0.44	0.022	0.37	0.027	0.44	0.030	0.38	0.019	0.39	0.013	0.671	0.410	0.048	0.147	0.085
20:3*n-3**	0.17	0.020	0.13	0.008	0.12	0.012	0.13	0.010	0.15	0.007	0.12	0.006	0.508	0.130	0.012	0.071	0.050
20:3*n-6**	0.41	0.035	0.43	0.025	0.36	0.034	0.38	0.029	0.41	0.026	0.33	0.018	0.280	0.526	0.117	0.008	0.975
20:4*n-6**	3.23	0.420	3.69	0.191	2.99	0.327	3.00	0.224	3.30	0.226	2.68	0.157	0.163	0.204	0.374	0.006	0.952
20:5*n-3**	0.10	0.021	0.11	0.009	0.07	0.009	0.09	0.012	0.08	0.006	0.06	0.006	0.092	0.708	0.056	0.006	0.373
22:4*n-6**	0.56	0.048	0.49	0.029	0.47	0.047	0.48	0.035	0.47	0.041	0.40	0.024	0.062	0.383	0.058	0.202	0.711
22:5*n-3**	0.40	0.065	0.36	0.018	0.31	0.032	0.31	0.023	0.32	0.033	0.25	0.029	0.035	0.789	0.087	0.031	0.776

22:6*n-3*[‡§]	0.17	0.026	0.19	0.025	0.09	0.015	0.16	0.015	ND	-	ND	-	0.326	-	-	-	-
Others[*]	3.77	0.439	4.60	0.300	3.94	0.375	3.78	0.291	4.35	0.277	3.80	0.172	0.621	0.053	0.801	0.038	0.916
Fatty acid partial sums																	
SFA[*]	35.2	0.467	33.1	0.581	34.1	0.457	34.3	0.436	33.5	0.464	34.1	0.312	0.636	0.006	0.130	0.087	0.338
MUFA[*]	39.7	1.56	41.1	0.92	44.0	1.27	41.2	0.82	41.9	1.04	45.0	0.61	0.220	0.364	0.001	0.003	0.943
PUFA[*]	21.6	1.52	21.2	0.87	17.8	1.27	20.9	0.89	20.0	0.92	17.1	0.49	0.311	0.597	0.002	0.001	0.964
n-6 PUFA[*]	20.3	1.43	20.1	0.86	16.9	1.21	19.7	0.86	19.1	0.88	16.3	0.47	0.388	0.690	0.003	0.001	0.965
n-3 PUFA[*]	1.28	0.097	1.12	0.047	0.91	0.066	1.13	0.046	0.93	0.051	0.74	0.029	0.001	0.009	<0.001	<0.001	0.934
Fatty acid ratios																	
PUFA/SFA[‡]	0.65	0.037	0.55	0.057	0.52	0.048	0.59	0.033	0.57	0.038	0.50	0.018	0.544	0.034	0.005	0.350	0.547
n-6/n-3	16.4[a]	0.43	18.3[bca]	0.73	18.4[c]	0.40	17.7[ca]	0.57	20.6[db]	0.57	22.1[d]	0.38	<0.001	<0.001	<0.001	0.147	0.031

NPD, normal crude protein diet; RPD, reduced crude protein diet; RPDL, reduced crude protein diet with Leu supplementation.
SFA = 12:0+14:0+15:0+16:0+17:0+18:0+20:0; MUFA = 16:1*c*7+16:1*c*9+17:1*c*9+18:1+18:1*c*9+18:1*c*11+20:1*c*11; PUFA = 18:2*n-6*+18:3*n-3*+20:2*n-6*+20:3*n-3*+20:3*n-6*+20:4*n-6*+20:5*n-3*+22:4*n-6*+22:5*n*-3+22:6*n-3*; *n-6* PUFA = 18:2*n-6*+20:2*n-6*+20:3*n-6*+20:4*n-6*+22:4*n-6*; *n-3* PUFA = 18:3*n-3*+20:3*n-3*+20:5*n-3*+22:5*n-3*+22:6*n-3*.
[a,b,c,d] Mean values within a row with unlike superscript letters were significantly different (P<0.05).
[*] Variable adjusted for IMF. [†] Variable adjusted for IMF × arginine × dietary protein level interaction. [‡] Variable adjusted for IMF × dietary protein level interaction. [§] Variable adjusted for IMF × arginine interaction.
ND – not detected.

Concerning partial sums of fatty acids (Table 5.3), the RPD decreased the percentage of SFA (*P*=0.006) when compared to the NPD diet. The proportion of MUFA was higher in the RPDL relative to the NPD and RPD. In contrast, the PUFA and *n-6* PUFA were lower in the RPDL when compared to the NPD and RPD. The PUFA/SFA ratio was lower in the RPD and RPDL when compared to the NPD, while a significant interaction between arginine supplementation and protein level (*P*=0.031) was obtained for the *n-6/n-3* ratio.

5.3.2. Total fat content and fatty acid composition of subcutaneous adipose tissue

Results of backfat thickness at P_2 site, total fat and fatty acid composition for SAT are presented in Table 5.4. Dietary arginine supplementation did not affect (*P*=0.720) backfat thickness at P_2 site. However, an increase of 18% and 21% of P_2 backfat thickness was observed for pigs fed RPD (*P*=0.041) and RPDL (*P*=0.021), respectively, when compared to animals fed NPD. Total fat of SAT of pigs fed arginine was 6% higher (*P*=0.020) when compared to the animals fed a diet without amino acid supplementation. The RPD (*P*=0.005) and RPDL (*P*=0.003) also had a 10% increase in total fat content when compared with NPD. The most representative fatty acids in SAT were 18:1*c*9 (37-40% of total FAME), 16:0 (21-22%), 18:2*n-6* (16-19%) 18:0 (11-12%) and 18:1*c*11 (2-3%) for all experimental groups. Three individual fatty acids were affected by the arginine and protein level interaction, including the predominant fatty acids 18:2*n-6*, 20:2*n-6* and the “others” detected fatty acids. Neither arginine nor leucine changed the fatty acid profile in SAT. Reduction in the level of dietary protein resulted in an increase of the percentages of 18:1*c*9 (RPD and RPDL) and 20:1*c*11 (RPDL) when compared with NPD. Regarding fatty acid partial sums (Table 4), a significant interaction between arginine and dietary protein level was observed for PUFA (*P*=0.029) and *n-6* PUFA (*P*=0.025), while a significant effect on NPD *vs.* RPD (*P*=0.021) and NPD *v*. RPDL (*P*=0.004) was found for MUFA, with higher values in pigs fed RPD and RPDL. In terms of fatty acid ratios, the RPD decreased PUFA/SFA ratio (*P*=0.040) when compared with the NPD. However, the pigs fed RPD (*P*=0.043) and RPDL (*P*=0.024) had higher *n-6/n-3* ratios when compared with pigs fed the NPD.

Table 5.4 - Effect of dietary arginine, leucine and protein levels on backfat thickness P_2 (mm), total fat (% fat), fatty acid composition (% total fatty acids), partial sums of fatty acids and related ratios of subcutaneous adipose tissue of pigs.

	Control						Arginine						Significance level				
	NPD		RPD		RPDL		NPD		RPD		RPDL		Arg	Dietary protein level			Arg×Prot
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE		NPD vs RPD	NPD vs RPDL	RPD vs RPDL	
P_2 backfat thickness	17.1	1.39	18.1	1.39	19.1	1.39	15.3	1.39	20.2	1.39	20.0	1.39	0.720	0.041	0.021	0.780	0.349
Total fat	61.4	2.02	64.2	2.02	65.3	2.02	61.7	2.02	70.6	2.02	70.6	2.02	0.020	0.005	0.003	0.804	0.280
Fatty acid composition																	
12:0	0.06	0.003	0.05	0.002	0.06	0.003	0.06	0.002	0.06	0.004	0.05	0.003	0.988	0.605	0.691	0.901	0.060
14:0*	1.09	0.025	1.07	0.018	1.06	0.022	1.06	0.028	1.13	0.037	1.04	0.027	0.867	0.348	0.448	0.075	0.297
15:0	0.06	0.005	0.05	0.004	0.06	0.007	0.07	0.009	0.05	0.006	0.05	0.006	0.707	0.037	0.077	0.830	0.663
16:0*	21.6	0.30	21.2	0.29	21.3	0.32	21.1	0.35	22.1	0.38	21.2	0.24	0.780	0.367	0.737	0.173	0.090
16:1*c*7*	0.39	0.022	0.38	0.022	0.37	0.019	0.44	0.033	0.35	0.013	0.36	0.019	0.901	0.054	0.073	0.898	0.165
16:1*c*9	1.88	0.105	1.76	0.105	1.81	0.119	1.68	0.075	1.79	0.102	1.66	0.078	0.197	0.959	0.638	0.693	0.480
17:0	0.52	0.039	0.44	0.032	0.47	0.046	0.53	0.060	0.45	0.030	0.45	0.028	0.985	0.064	0.131	0.766	0.841
17:1*c*9	0.43	0.028	0.35	0.030	0.38	0.035	0.40	0.040	0.37	0.033	0.35	0.024	0.629	0.090	0.144	0.774	0.658
18:0	11.5	0.33	11.3	0.54	11.3	0.53	11.4	0.48	12.4	0.46	11.8	0.34	0.120	0.478	0.822	0.629	0.392
18:1	0.09	0.006	0.09	0.010	0.08	0.005	0.08	0.003	0.09	0.005	0.08	0.005	0.311	0.502	0.587	0.297	0.577
18:1*c*9	38.0	0.39	39.2	0.62	39.1	0.54	37.1	0.35	39.0	0.48	39.9	0.64	0.769	0.002	<0.001	0.521	0.239
18:1*c*11	2.60	0.055	2.54	0.111	2.53	0.115	2.45	0.070	2.47	0.078	2.48	0.064	0.199	0.757	0.784	0.972	0.781
18:2*n-6**	17.7^{ab}	0.42	17.7^{ab}	0.62	17.5^{ab}	0.33	19.5^{b}	0.76	16.5^{a}	0.33	16.9^{ab}	0.79	0.956	0.010	0.030	0.815	0.030
18:3*n-3*	1.05	0.045	0.94	0.040	0.95	0.020	1.11	0.054	0.88	0.026	0.85	0.049	0.300	<0.001	<0.001	0.882	0.226
20:0	0.23	0.009	0.21	0.012	0.20	0.007	0.20	0.009	0.19	0.008	0.20	0.009	0.058	0.245	0.358	0.707	0.247
20:1*c*11	0.76	0.036	0.81	0.055	0.82	0.025	0.73	0.050	0.72	0.035	0.84	0.039	0.309	0.653	0.036	0.123	0.415
20:2*n-6**	0.79^{ab}	0.023	0.87^{ab}	0.038	0.83^{ab}	0.020	0.83^{ab}	0.040	0.73^{a}	0.031	0.84^{b}	0.028	0.302	0.688	0.421	0.189	0.011
20:3*n-3*	0.11	0.005	0.11	0.006	0.12	0.007	0.12	0.011	0.09	0.006	0.10	0.007	0.237	0.025	0.385	0.109	0.143
20:3*n-6*	0.31	0.015	0.28	0.015	0.30	0.013	0.35	0.033	0.27	0.007	0.28	0.020	0.813	0.006	0.058	0.305	0.406
20:4*n-6*	0.15	0.007	0.13	0.015	0.13	0.010	0.16	0.015	0.13	0.007	0.15	0.013	0.285	0.054	0.191	0.508	0.924
Others	0.46^{ab}	0.052	0.49^{ab}	0.037	0.71^{b}	0.081	0.48^{ab}	0.054	0.42^{ab}	0.059	0.42^{a}	0.048	0.017	0.731	0.136	0.069	0.046

Fatty acid partial sums																	
SFA	34.8	0.61	34.2	0.79	34.3	0.80	34.2	0.79	36.7	0.70	35.3	0.51	0.119	0.194	0.658	0.363	0.110
MUFA	44.2	0.49	45.2	0.77	45.1	0.77	42.9	0.41	44.7	0.61	45.6	0.66	0.461	0.021	0.004	0.578	0.318
PUFA*	20.1^{ab}	0.47	20.0^{ab}	0.65	19.8^{ab}	0.36	22.1^{b}	0.87	18.6^{a}	0.36	19.1^{ab}	0.86	0.885	0.006	0.025	0.740	0.029
n-6 PUFA*	19.0^{ab}	0.43	18.9^{ab}	0.61	18.8^{ab}	0.34	20.8^{b}	0.82	17.6^{a}	0.35	18.1^{ab}	0.81	0.937	0.009	0.033	0.732	0.025
n-3 PUFA	1.16	0.046	1.05	0.043	1.07	0.023	1.23	0.062	0.97	0.027	0.96	0.053	0.271	<0.001	<0.001	0.885	0.170
Fatty acid ratios																	
PUFA/SFA*	0.58	0.022	0.59	0.026	0.58	0.022	0.65	0.041	0.51	0.020	0.55	0.031	0.682	0.040	0.136	0.545	0.050
n-6/n-3	16.8	0.48	18.3	0.43	17.6	0.21	17.3	0.54	17.8	0.45	18.7	0.54	0.290	0.043	0.024	0.838	0.188

NPD, normal CP diet; RPD, reduced CP diet; RPDL, reduced CP diet with Leu supplementation.

SFA = 12:0+14:0+15:0+16:0+17:0+18:0+20:0; MUFA = 16:1*c*7+16:1*c*9+17:1*c*9+18:1+18:1*c*9+18:1*c*11+20:1*c*11; PUFA = 18:2*n-6*+18:3*n-3*+20:2*n-6*+20:3*n-3*+20:3*n-6*+20:4*n-6*; *n-6* PUFA = 18:2*n-6*+20:2*n-6*+20:3*n-6*+20:4*n-6*; *n-3* PUFA = 18:3*n-3*+20:3*n-3*.

[a,b] Mean values within a row with unlike superscript letters were significantly different ($P<0.05$).

* Variables adjusted for total fat.

5.3.3. Gene expression levels of muscle and subcutaneous adipose tissue

The results presented above showed different responses of the *longissimus lumborum* muscle and SAT to dietary manipulations in crossbred pigs. Analysis of expression of key genes associated with lipid metabolism has been carried out in order to elucidate whether the tissue-specific effects of dietary arginine, leucine and protein level are associated with modulation of the gene expression. The Figures 5.1 and 5.2 show the expression levels of 14 key genes controlling lipid metabolism analysed in *longissimus lumborum* muscle and SAT of pigs, respectively.

In *longissimus lumborum* muscle, the expression level of 9 of the 14 genes was affected, at least, by one dietary treatment. A significant interaction (P=0.008) between arginine and protein level was found for the expression level of *FADS1* gene. Arginine increased the *FABP4* (P=0.026), *FASN* (P=0.030) and *SREBP1* (P=0.001) mRNA levels, and decreased the expression levels of *MLXIPL* (P=0.001) and *PPARγ* (P=0.041) genes. RPDL decreased the expression levels of *MLXIPL* (P=0.004) and *PPARα* (P=0.016), and increased *FASN* mRNA (P=0.031), when compared with the NPD. The relative expression level of *LPL* gene was higher (P=0.043) in pigs fed the RPD relative to the NPD. Finally, the RPD (P=0.024) and RPDL (P=0.006) increased the relative expression levels of the *SCD* gene when compared with the NPD.

In SAT, the mRNA levels of 11 of the 14 genes analysed were affected by, at least, one dietary treatment. A significant interaction between arginine and dietary protein was found for *FABP4* (P=0.026), *PPARγ* (P=0.009) and *SREBP1* (P=0.044). Arginine increased the *FASN* (P=0.022) and *SCD* (P=0.016) mRNA expression. The RPD increased the expression levels of the *FASN* (P=0.049) and *LPL* (P=0.004) when compared with the NPD. The RPDL increased the mRNA levels of the *FADS1* (P<0.05), *FADS2* (P<0.05) and *SCD* (P<0.01) when compared with NPD and RPD. The expression levels of *CEBPα* (P=0.002) and *CRAT* (P=0.043) genes were down-regulated, and that of *ACACA* gene (P=0.011) up-regulated, in pigs fed RPDL, when compared with the animals fed NPD. Finally, the RPDL increased the SCD mRNA levels when compared with NPD (P<0.001) and RPD (P=0.008).

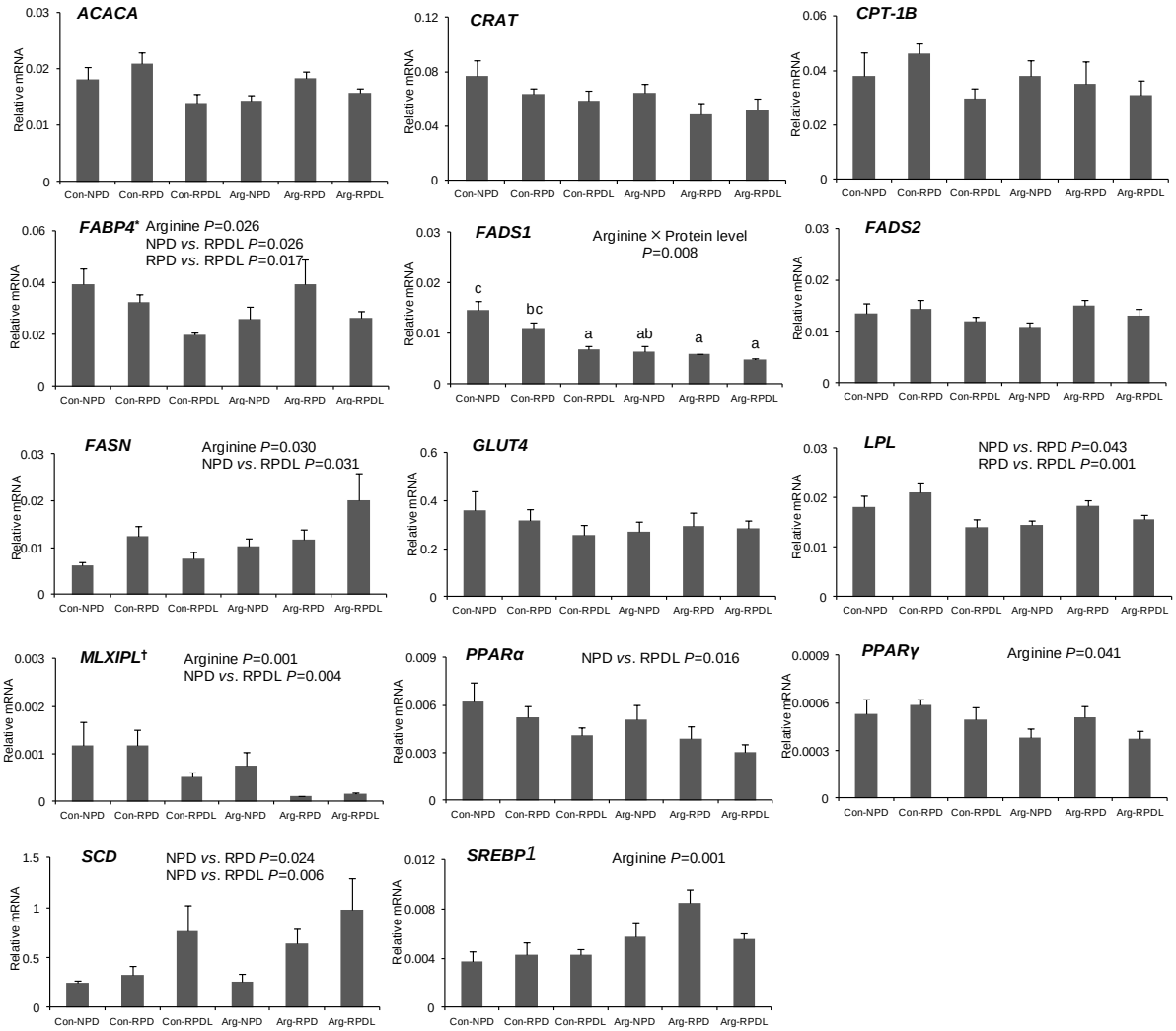

Figure 5.1 - Effect of dietary arginine, leucine and protein levels on gene expression in *longissimus lumborum* muscle of pigs. Con, control diet; NPD, normal protein diet; RPD, reduced protein diet; RPDL, reduced protein diet with leucine aupplementation. [a,b] Mean values within a row with unlike superscript letters were significantly different ($P<0.05$). * Variable adjusted for IMF × Arginine interaction. † Variable adjusted for IMF.

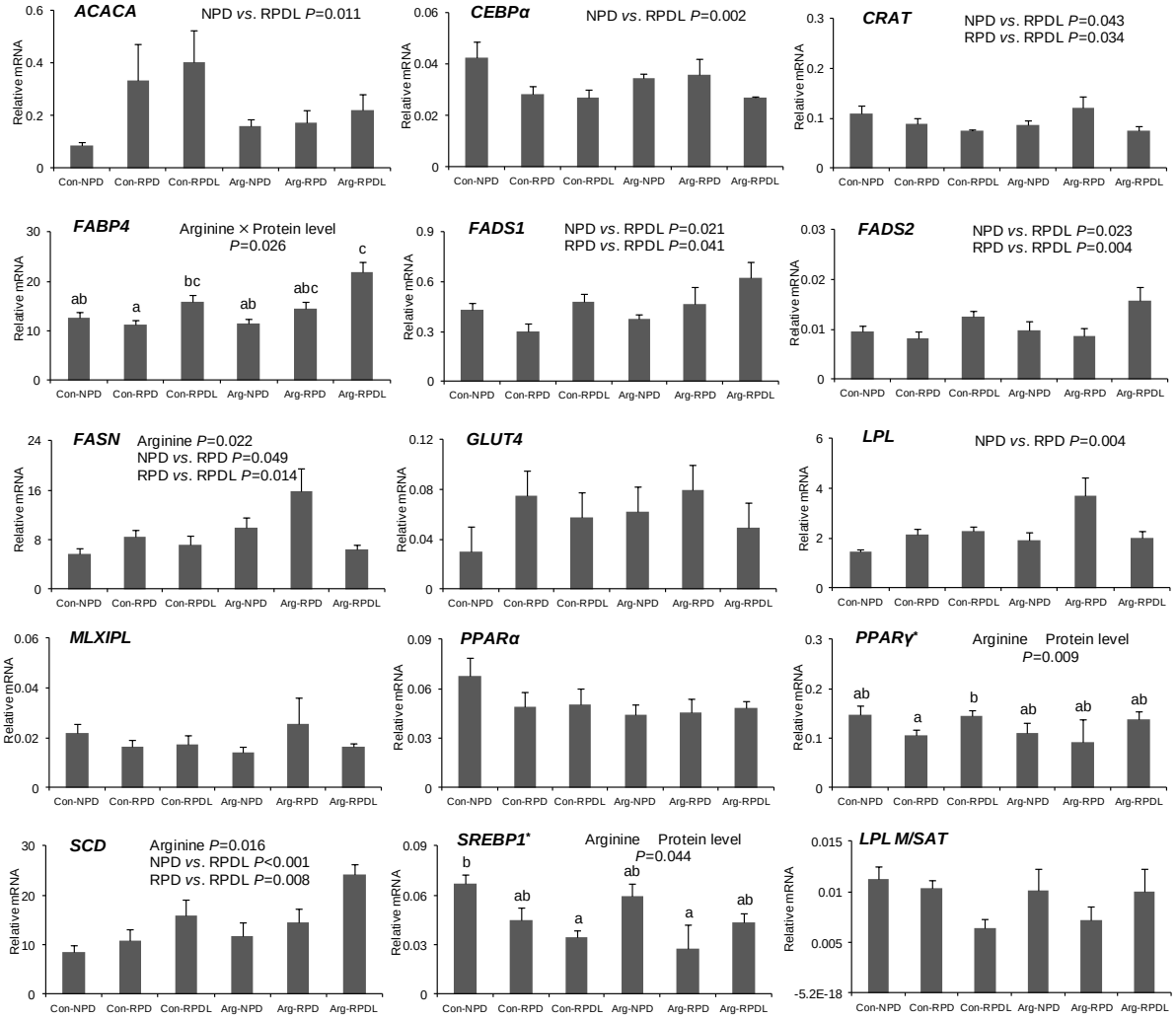

Figure 5.2 - Effect of dietary arginine, leucine and protein levels on gene expression in subcutaneous adipose tissue of pigs. Con, control diet; NPD, normal protein diet; RPD, reduced protein diet; RPDL, reduced protein diet with leucine supplementation. [a,b] Mean values within a row with unlike superscript letters were significantly different (P<0.05). [*] Variables adjusted for Total fat × Arginine × Reduced protein interaction.

5.3.4. Correlation between fatty acid composition and gene expression levels

Table 5.5 presents correlation coefficients (r) for fatty acid composition and gene expression levels, adjusted with IMF as covariate for *longissimus lumborum* muscle, and fatty acid composition with total fat as covariate for SAT. There were a much larger number of significant correlations between fatty acid composition and gene expression in SAT relative to the muscle.

In *longissimus lumborum* muscle, only 18:0 fatty acid (P<0.05) was positively and moderately correlated (0.7≥r≥0.3) with *FADS1*. Strong positive correlations (P<0.001) were observed between *FASN* and *ACACA*, and between *FASN* and *SCD*. Expressions of *PPARα* (P<0.001) and *MLXIPL* (P<0.001) were positively correlated with *CPT-1B*, *CRAT* and *FADS1.* Furthermore, positive correlations were found between *PPARα* expression and

Table 5.5 - Pearson´s correlation coefficients among fatty acid composition (% total fatty acids) and gene expression levels (relative mRNA level) in *longissimus lumborum* muscle and subcutaneous adipose tissue of pigs.

		16:0	16:1c9	18:0	18:1c9	18:1c11	18:2n-6	SFA	MUFA	PUFA	*SREBP1*	*SCD*	*PPARγ*	*PPARα*	*MLXIPL*	*LPL*	*GLUT4*	*FASN*	*FADS2*	*FADS1*	*FABP4*	*CRAT*	*CPT-1B* *CEBPα*	*ACACA*
Longissmus lumborum muscle	*ACACA*	-0.20	-0.17	0.11	-0.08	-0.20	0.11	-0.10	-0.10	0.11	0.25	0.18	0.21	0.27	0.19	0.35*	0.05	0.56***	0.04	0.21	-0.16	0.19	0.01	
	CPT-1B	0.09	-0.20	0.23	-0.04	-0.14	0.01	0.12	-0.07	0.01	0.29*	0.09	0.17	0.68***	0.48***	0.40**	0.43**	-0.04	-0.05	0.26	-0.23	0.71***		
	CRAT	0.06	-0.20	0.26	-0.13	-0.14	0.14	0.10	-0.14	0.12	0.22	0.18	0.30*	0.86***	0.61***	0.42**	0.62***	0.03	0.11	0.46***	-0.20			
	FABP4	-0.01	0.02	0.06	-0.04	0.01	0.06	0.06	-0.03	0.04	-0.00	-0.21	0.41**	-0.11	0.16	0.20	-0.14	-0.05	0.27	0.22				
	FADS1	0.07	-0.22	0.30*	-0.25	-0.10	0.19	0.23	-0.25	0.19	-0.03	-0.18	0.35*	0.62***	0.65***	0.48***	0.20	-0.13	0.44**					
	FADS2	0.03	0.04	0.01	0.16	0.19	-0.12	0.07	0.16	-0.13	0.21	0.09	0.23	0.22	0.31*	0.35*	0.16	0.07						
	FASN	-0.04	-0.06	-0.08	0.17	-0.02	-0.13	-0.08	0.13	-0.13	0.05	0.70***	0.21	-0.04	-0.07	0.28	-0.00							
	GLUT4	0.05	0.09	0.04	-0.08	0.18	0.03	-0.02	-0.05	0.03	0.16	0.12	0.24	0.46***	0.41**	0.37*								
	LPL	0.08	0.05	0.13	0.14	0.19	-0.16	0.08	0.14	-0.15	0.21	0.11	0.47***	0.42**	0.48**									
	MLXIPL	0.02	-0.18	0.26	-0.05	-0.10	0.08	0.12	-0.06	0.06	0.06	-0.12	0.33*	0.61***										
	PPARα	-0.06	-0.25	0.21	-0.21	-0.18	0.23	0.03	-0.23	0.21	0.27	0.05	0.28*											
	PPARγ	-0.01	0.10	-0.04	0.03	0.17	-0.09	-0.07	0.05	-0.04	0.01	0.00												
	SCD	0.02	-0.05	-0.15	0.04	0.02	-0.10	-0.06	0.01	-0.07	0.20													
	SREBP1	0.05	0.10	0.01	0.05	0.05	-0.09	0.04	0.04	-0.09														
Subcutaneous adipose tissue	*ACACA*	0.01	-0.06	0.08	0.13	-0.03	-0.14	0.05	0.09	-0.14	-0.10	0.36*	0.10	0.08	0.20	0.16	0.50***	0.18	0.17	0.23	0.04	-0.11	-0.21	
	CEBPA	0.15	-0.04	0.20	-0.43**	-0.14	0.19	0.23	-0.41**	0.18	0.19	-0.13	0.06	0.16	0.30*	0.06	0.01	0.21	0.00	0.15	-0.17	0.09		
	CRAT	0.40**	-0.07	0.32*	-0.15	-0.20	-0.20	0.38**	-0.17	-0.21	0.30*	-0.07	0.30*	0.37*	0.47***	0.51***	0.15	0.52***	0.01	0.18	0.06			
	FABP4	0.09	-0.08	0.22	0.19	-0.11	-0.27*	0.23	0.13	-0.28*	-0.10	0.38**	0.26	-0.01	0.14	0.20	-0.08	-0.01	0.37**	0.30*				
	FADS1	0.13	0.00	0.16	0.14	0.04	-0.25	0.20	0.11	-0.23	0.27	0.52***	0.37**	0.29*	0.47***	0.33*	0.29*	0.38**	0.61***					
	FADS2	0.07	-0.07	0.16	0.23	-0.07	-0.29*	0.13	0.16	-0.28*	0.22	0.48***	0.16	0.30*	0.32*	0.09	0.29*	0.06						
	FASN	0.20	-0.24	0.39**	-0.07	-0.27	-0.16	0.39**	-0.14	-0.18	0.15	0.47**	0.18	0.11	0.61***	0.70***	0.84***							
	GLUT4	-0.10	-0.25	0.18	0.03	-0.22	-0.03	0.08	-0.04	-0.03	0.14	0.55***	0.10	0.22	0.60***	0.56***								
	LPL	0.31*	-0.08	0.38**	-0.01	-0.23	-0.29*	0.44**	-0.08	-0.31*	0.03	0.16	0.42**	0.19	0.53***									
	MLXIPL	0.14	-0.14	0.28*	-0.14	-0.19	-0.07	0.28*	-0.17	-0.07	0.24	0.08	0.38**	0.99***										
	PPARα	0.10	-0.08	0.22	-0.13	-0.19	-0.03	0.20	-0.16	-0.03	0.34*	0.05	0.24											
	PPARγ	0.09	-0.05	0.13	-0.15	-0.11	-0.01	0.11	-0.16	-0.01	0.40**	0.01												
	SCD	0.04	-0.19	0.31*	0.37**	-0.13	-0.42**	0.28	0.25	-0.42**	0.04													
	SREBP1	-0.02	-0.09	0.08	-0.20	-0.03	0.11	-0.02	-0.18	0.12														

* $P<0.05$, ** $P<0.01$, *** $P<0.001$.

GLUT4 (*P*<0.001); *PRARα* and *MLXIPL* (*P*<0.01); *PPARγ* and *LPL* (*P*<0.001), *LPL* and *FADS1* (*P*<0.001). *GLUT4* was positively correlated with *CRAT* (*P*<0.001), while *FADS1* was positively correlated with *CRAT* (*P*<0.001). Finally, the *CRAT* gene expression was positively and highly correlated (r>0.7) with the *CPT-1B* (*P*<0.001).

In SAT, 16:0 (*P*<0.01), 18:0 (*P*<0.01) and SFA (*P*<0.01) were positively and moderately correlated with *CRAT* and *LPL* gene expression. Furthermore, 18:0 and SFA were also positively correlated with *FASN* (*P*<0.01). Moderate positive correlation was found between 18:0 and *SCD* gene expression (*P*<0.05). MUFA (*P*<0.01) and 18:1*c*9 (*P*<0.01) were negatively and moderately correlated with *CEBPα*, and 18:1*c*9 was positively correlated with *SCD* (*P*<0.01). The 18:2*n-6* and PUFA were negatively correlated with *FABP4* (*P*<0.05), *FADS2* (*P*<0.05), *LPL* (*P*<0.05) and *SCD* (*P*<0.01). Significantly positive correlations in the SAT were observed between *SCD* and *ACACA* (*P*<0.01), *FABP4* (*P*<0.01), *FADS1 (P*<0.001*)*, *FADS2* (*P*<0.001), *FASN* (*P*<0.01) and *GLUT4 (P*<0.001*)* gene expression. It was also found that *PPARα* was positively correlated with *CRAT* (*P*<0.05), *FADS1* (*P*<0.05), *FADS2* (*P*<0.05) and *MLXIPL (P*<0.001), whilst *MLXIPL* was positively correlated with *CEBPA* (P<0.05), *CRAT (P<0.001)*, *FADS1 (P<0.001)*, *FADS2* (*P*<0.05), *FASN (P*<0.001*)*, *GLUT4 (P*<0.001*)* and *LPL (P*<0.001*)*. *LPL* and *FASN* were positively correlated with *CRAT (P*<0.001) and *FADS1* (*P*<0.05), and *LPL* was also positively correlated with *FASN (P*<0.001*)* and *GLUT4 (P*<0.001*)*. In addition, *GLUT4* was positively correlated with *ACACA (P*<0.001), *FADS1* (*P*<0.05), *FADS2* (*P*<0.05) and *FASN (P*<0.001). Finally, *FADS2* was positively correlated with *FABP4* (*P*<0.01) and *FADS1* (*P*<0.001).

5.4. Discussion

In the present study, a 19% RPD (16% *vs.* 13%) fed during the growing-finishing phase of the commercial crossbred (lean) pigs resulted in 45-48% of increase in IMF. This is consistent with findings of previous studies which demonstrated that range of protein concentrations (*e.g*. 21 *vs*. 18% (Doran *et al*., 2006); 17 *vs*. 15% (Alonso *et al*., 2010)) increased IMF content in commercial crossbred pigs. . It was also recently shown by our group that dietary lysine restriction (0.7% *v.* 0.4% of L-lysine) is needed to increase IMF in commercial crossbred pigs, and that the IMF increase is mediated via up-regulation of the *SCD* gene expression (Madeira *et al.*, 2013b, chapter 3), which encodes for the key lipogenic enzyme of MUFA biosynthesis. The results of present work confirm the up-regulation of *SCD* gene expression by RPD in pig muscle.

Our initial hypothesis was that tissue-specific responses to RPD in terms of fat content and fatty acid composition in crossbred pigs could be increased by dietary supplementation of arginine and leucine. This hypothesis was based on data of the literature, which suggested

that dietary supplementation of arginine (Tan *et al.*, 2009; Ma *et al.*, 2010) and leucine (Cisneros *et al*., 1996; Hyun *et al*., 2003 and 2007) can result in increased IMF content. We hypothesized that if these feeding strategies increase IMF through different mechanisms, they could have additive effects on IMF levels. We also proposed that this partitioning effect would be mediated via the differential expression of genes controlling lipid metabolism in muscle and SAT. The present study addressed the afore-mentioned aspects and the results suggested that neither arginine nor leucine has any additional increase of IMF in *longissimus lumborum* muscle.

Results of our study on effects of arginine supplementation are in line with that of Go *et al.* (2012), who found that dietary arginine supplementation does not increase IMF in pigs. However, Tan *et al.* (2009) and Ma *et al.* (2010) reported an increase in IMF content in experiments that used 1% of dietary arginine supplementation. This discrepancy might be explained by the use of pigs with distinct genetic background, mainly different predisposition for fat deposition, which determines the optimal protein level in diets and the IMF content. In fact, our previous results showed that the increase in IMF under the RPD depends on the genetic background of pigs (Madeira *et al.*, 2013b, chapter 3). In particular, the IMF increase under the RPD was observed in genetically lean but not in fatty pig breeds. Furthermore, Tan *et al*. (2009), who used Duroc × Large White × Landrace crossbred pigs fed with protein and arginine levels similar to those reported in the present study, observed higher IMF contents when compared with the animals from our control group (1.81% *vs*. 1.34%). In addition, Ma *et al*. (2010) fed Duroc × (Chang × Da) crossbred pigs with dietary protein and arginine levels similar to those described in this experiment and got IMF contents similar to ours in control animals (1.31% *vs.* 1.34%) but, in contrast to our data, a significant IMF increase with arginine supplementation.

Regarding the dietary leucine supplementation of RPD, our results are in disagreement with those of Hyun *et al*. (2007), who described increased IMF with leucine supplementation of diets restricted in lysine. Since the levels of crude protein and lysine were similar in both studies, the difference between results of our study and the data of Hyun *et al.* (2007) might be explained by the use of a different genetic background (PIC line 327 × C22). It is also important to point out that Hyun *et al.* (2007) studied the effect of dietary leucine supplementation on a normal protein diet and not on a RPD, as used in our experiment. In sum, our results do not indicate any additional effect of dietary arginine and/or leucine supplementation on increased IMF promoted by the RPD alone.

In terms of dietary effects on fatty acid composition, the animals fed the diet supplemented with arginine had a lower percentage of *n-3* long-chain PUFA, which was mainly due to a decrease in DPA level. The DPA decrease was accompanied by the lower expression of *FADS1* and *MLXIPL* genes and the higher expression of *SREBP1* gene. The Δ5-desaturase

is an enzyme directly involved in the biosynthesis of long-chain PUFA (Glaser *et al*., 2010) It is possible that changes in the Δ5-desaturase gene expression (*FADS1* gene) and activity are linked to the changes in the level of transcription factors *SREBP1* and *MLXIPL* observed in this study. In fact, our observation of the lower concentration of PUFA in muscle membranes and the up-regulation of *SREBP1* under arginine-supplemented diet are consistent with the central role of *SREBP1* in allostatic regulation of membrane lipid composition proposed by y Hagen, Rodriguez-Cuenca & Vidal-Puig (2010). The *MLXIPL* also participate in regulation of lipogenic enzymes expression, and it has been described to act synergistically with *SREBP1* (Dubuquoy *et al*., 2011).

In contrast to *FADS1* gene, our study did not find significant effects of arginine on *SCD* gene expression. This is not in agreement with findings of Tan *et al*. (2011) who reported that arginine supplementation increased the 18:1*c*9 and decreased 18:0 and 18:2*n-6*, which was explained by arginine-dependent activation of *SCD*, a key enzyme in the formation of oleic acid. These differences might be explained by variations in the experimental conditions, mainly by the different genetic line of pigs used (Duroc × Large White × Landrace), which determines distinct IMF levels for control animals.

Our study indicated that supplementation of RPD with leucine changed the fatty acid profile in pig muscle. The up-regulation of *SCD* (*P*=0.09) and *FASN* in muscle were consistently reflected by higher proportions of 18:1*c*9 and 16:0 fatty acids. Moreover, most of the muscle PUFA decreased under the dietary leucine supplementation. As the supplementation of RPD with leucine did not increase IMF (*P*=0.780), it is reasonable to assume that the proportion of membrane phospholipids and triacylglycerols in muscle remains fairly constant. Thus, the increase in 18:1*c*9 and 16:0 should result in a direct replacement of the majority of PUFA in muscle lipids. Furthermore, the expression of transcription factors *MLXIPL* and *PPARα*, as well as *FABP4* expression, were down-regulated by the leucine supplementation, which is not easy to relate with the apparent replacement of PUFA by 16:0 and 18:1*c*9. In addition, expression of *PPARα* in muscle was noted to be up-regulated in pigs fed the NPD when compared with pigs fed the RPDL, thus suggesting enhanced lipid oxidation.

In the present study, RPDs increased total fat in SAT and backfat thickness in pigs. Michal *et al*. (2006) reported that FABP4 protein is responsible for the transport of fatty acids in adipocytes and its content is associated with backfat thickness. However, in this study, the *FABP4* mRNA levels did not increase in pigs fed RPD. Also, this diet increased 18:1*c*9 and MUFA content, which may be explained by a higher expression of the gene controlling lipogenesis (*FASN*) and expression of the *LPL*.

This study established that dietary arginine supplementation increased total fat content in SAT, without effect on fatty acid composition and backfat thickness at the P_2 site. The increased fat content of SAT is consistent with the up-regulation expression of *FASN* and

SCD genes in animals fed an arginine-supplemented diet. However, these data do not support the results of previous works, in which arginine was suggested to increase lipolysis and to inhibit lipogenesis in adipose tissue of pigs (Tan *et al*., 2009), rats (Fu *et al*., 2005) and broilers (Fouad, El-Senousey, Yang & Yao, 2013). This discrepancy of results in pigs might be explained by the genetic line used by Tan *et al.* (2009), which was different to the genetic background used in this (Duroc × Large White × Landrace *vs.* Duroc × Large White × Landrace × Pietrain). However, the crude protein and lysine levels used are similar in the two experiments. Also, Tan *et al*. (2011) reported a decrease of 18:1*c*9 in SAT with arginine supplementation, which was not confirmed by our results.

In SAT, leucine supplementation of RPD did not affect total fat content, fatty acid composition and backfat thickness at P_2 site. However, leucine induced extensive modification of the gene expression pattern, with up-regulation of *ACACA*, *FADS1*, *FADS2* and *SCD*, and down-regulation of *FASN*, *CEBPα* and *CRAT*, although this mRNA level changes seem to be unrelated to the fatty acid composition of adipose tissue. The *MLXIPL* mRNA expression levels were strongly correlated with *GLUT4* expression. It is known that the transcription factor *MLXIPL* is highly regulated by *GLUT4* in adipose tissue and it is a key determinant of systemic insulin sensitivity (Herman *et al*., 2012). Despite the lack of differences in the mRNA levels of GLUT4 among experimental groups, in both muscle and SAT, it is clear that *GLUT4* gene is more expressed in muscle than in SAT, thus suggesting that energy substrates are preferentially used by skeletal muscle rather than white adipose tissue. Finally, our results indicated a negative correlation between the expression levels of *FADS2* and the percentages of 18:2*n*-6 and PUFA in SAT.

Comparing the expression level of genes controlling lipid metabolism in muscle and SAT, the mRNA levels of *ACACA*, *CRAT*, *FABP4*, *FASN*, *LPL*, *PPARα, PPARγ* and *SCD* were higher in SAT than in muscle. Furthermore, this study found a higher degree of correlation between the percentage of major fatty acids and partial sums of fatty acids with the expression level of key lipogenic enzymes and transcription factors in SAT than in muscle. This observation might be explained by the distinct roles of these two tissues in pig lipid metabolism. In fact, while SAT is a main site for *de novo* fatty acid biosynthesis and lipogenesis, muscle plays a major role in the metabolism of glucose and degradation of lipids (Bergen & Mersmann, 2005).

5.5. Conclusions

This study indicates that dietary arginine supplementation does not have a significant effect on IMF content in pigs, but increases total fat content in SAT without change backfat thickness at the P_2 site. However, gene expression analysis suggests that arginine decreases the mRNA level of some lipogenic genes in muscle. The increased total fat content in SAT seems to be mediated by the up-regulation of *FASN* and *SCD* mRNA expression. Therefore, arginine might be involved in the differential regulation of some key lipogenic genes expression in pig muscle and SAT. In addition, the data confirmed that RPD with restricted lysine level increases IMF content, total fat content in SAT and backfat thickness at the P_2 site. Moreover, leucine supplementation of RPD does not seem to result in additional increase of IMF, total fat in SAT or backfat thickness at the P_2 site. In spite of the lack of effect of leucine supplementation on fat content, the leucine-supplemented diet increased the expression of some genes encoding for lipogenic enzymes, namely *FASN* and *SCD* in muscle, and *FADS1*, *FADS2* and *SCD* in SAT.

Arginine supplementation seems to affect only *n-3* PUFA in muscle, in contrast to RPD and leucine supplementation of RPD, which changed the percentage of most of fatty acids. The increase of MUFA content in muscle of pigs fed RPD and RPD with leucine supplementation seems to be cumulative due to the additional *SCD* mRNA expression. In SAT, only RPD seems to change fatty acid composition, which is likely mediated by the increase of *FASN* and *LPL* mRNA expression.

Therefore, under our experimental conditions, arginine supplementation of pig diets, either alone or in combination with RPD and/or leucine, does not seem to be useful to increase IMF content or to change fatty acid composition of pork. In contrast, the supplementation of RPD with leucine seems to be interesting to increase MUFA content in pork. The results of this study also indicate that adipogenesis and lipogenesis might be differently regulated in pig *longissimus lumborum* muscle and SAT. These data contribute to understand the mechanisms of dietary regulation of fat partitioning in pigs and, therefore, could help to improve pig feeding strategies to address industry needs and consumers' demands.

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Chapter 6

GENERAL DISCUSSION, CONCLUSIONS, IMPLICATIONS AND FUTURE PERSPECTIVES

6.1. General discussion

The major aim of pig industry is to provide meat with quality to the consumers and, simultaneously, to improve the productive efficiency in order to low production costs. However, due to the genetic selection practiced by industry in commercial breeds, pork has become increasingly leaner and the amount of IMF in *longissimus dorsi* muscle from commercial breeds is very low (less than 2%) (Tomasz, Tomasz & Jerzy, 2005). It is well established that acceptable pork eating quality requires a minimum IMF of 2.5%. Reduction in IMF below 2% affects negatively the palatability, juiciness and tenderness of pork (De Vol *et al.*, 1988, Eikelenboom *et al.*, 1994a). Therefore, there is a need to increase the IMF content to improve meat quality traits, mainly the sensory attributes of pork, without increase SAT and without affect productive traits of pigs. In line with this, some studies have been developed feeding strategies to improve IMF content. The studies reported in this thesis, based on two pig experiments, aimed to test some feeding strategies based on RPD and/or amino acid supplementation to improve pork quality traits, mainly sensory traits and fatty acid composition, as well as to explore the metabolic and molecular mechanisms associated with carcass fat partitioning.

6.1.1. RPDs and lysine restriction effects on the Alentejano and crossbred Pigs

In the first pig experiment we used two distinct genetic lines of pigs in the growing-finishing phase of growth: a) Alentejano purebred, a Portuguese autochthonous breed, which was used as a high-fat porcine genotype with a slow protein deposition rate; and b) a commercial crossbred pig genotype. This crossbred pig was a result of the three genetic lines, 50% Large White × 50% Landrace gilts mated to 50% Large White × 50% Pietrain boars, that result in 50% Large White × 25% Landrace × 25% Pietrain, a commercial low-fat porcine genotype with fast protein deposition rate.

It is well known that RPDs tend to increase pork IMF content, although some inconsistent responses have been reported. Therefore, in this experiment, our main objective was to investigate if the effect of RPDs on IMF was due the protein reduction or lysine reduction that accompanies the protein reduction. Then, we used three isoenergetic diets (13.5 MJ ME/kg), a control diet and two RPDs with 23% less of crude protein. A control diet with 17.5% of crude protein and 0.65% lysine; a RPD supplemented with lysine added with 13.2% of crude protein and 0.56% lysine; and a RPD not adjusted for lysine with 13.1% of crude protein and 0.40% lysine. There was no difference in the lysine content between the control and RPD supplemented with lysine. The lysine content of RPD was 38.5% less than in the control diet.

The main objective of using RPDs in the growing-finishing phase of pigs is to increase IMF content and, consequently, to improve meat quality mainly the sensory characteristics without affect SAT and productive traits. Also, previous studies reported that the molecular mechanisms associated to the increase IMF with RPDs was the increased SCD protein expression in pig muscle but not in SAT (Doran *et al*., 2006). This work concludes that SCD protein expression in muscle correlates with the level of intramuscular fatty acids. As well, Ntawubizi *et al*. (2009) reported that pigs with a lower IMF content have lower *de novo* fatty acid synthesis. Guo *et al.* (2011) demonstrated that the expression of lipogenic enzymes, mainly SCD, have a significant impact on IMF deposition in pigs. Our results showed that the RPD without lysine adjustment increased the IMF, which is accompanied of the up-regulation of SCD content in crossbred pigs, but not in Alentejano pigs. It is well known that protein deposition requires both energy and amino acids. If the amino acid intake is limiting, increasing the energy intake will be directed to fat synthesis (Pettigrew & Esnaola, 2001).

Regarding the sensory traits, the Alentejano purebred had higher scores for tenderness, juiciness and flavour attributes. These results could be explained by the higher levels of IMF of Alentejano pigs. Wood *et al.* (2003) reported that lipids in the form of IMF tend to increase the amount of perceived moisture in muscle, thus improving juiciness in pork. There were no differences for diet in tenderness in our results. In contrast, Wood *et al.* (2004) reported that a low protein diet produces meat more tender. However, RPD not adjusted for lysine increased juiciness. This result was in accordance with Blanchard *et al.* (1999) and Wood *et al.* (2004), who found that low protein level increases juiciness. Lebret (2008) reported that an increase of IMF proportion improve meat tenderness and juiciness can be achieved with feeding pigs *ad libitum* with protein or lysine deficient, but sufficient energy diets during the growing or finishing phases. In general an increase level of IMF has a positive influence on the sensory qualities of pig meat (Fernandez *et al.*, 1999a).

The distribution of fat between different fat depots might be controlled by different mechanisms and possibly by different genes (Doran *et al.*, 2006; Mourot, Kouba & Bonneau, 1996). In our study, the *SCD* expression in muscle increased in crossbred pigs fed the RPD without lysine adjustment and a similar trend was observed for *PPARγ* expression. Doran *et al.* (2006) in their study reported that the positive correlation between SCD protein expression and the amount of total fatty acids in muscle suggest that SCD plays a significant role and it might be a candidate gene for intramuscular lipids development in the pig. Also, Wang *et al.* (2012) finding an increase of IMF and of *SCD* expression in muscle of pigs fed low protein diets at 100 kg of live weight. It is well know that ∆9 desaturase catalyses the conversion of SFA in MUFA (16:0 and 18:0 to 16:1 and 18:1, respectively).

In SAT, the principal site where occurs *de novo* fatty acid synthesis, the total fatty acid content increase with RPDs. These results are consistent with the data of Doran *et al.* (2006), who described that feeding the RPD increased the total fatty acid content of the adipose tissue by 8.5%, when compared with the control diet.

The RPDs decreased the PUFA and maintained the SFA and MUFA proportions in SAT. Contrarily, Doran *et al.* (2006) found an increase of 16:0, 18:0 and 18:1*c*9 in SAT with RPD. Therefore, the SCD activity in SAT was not affected by RPD and similar results were found by Doran *et al.* (2006). This fact can be possible due to a different SCD gene isoforms in muscle and SAT or other possible explication is that enzymes may be regulated differently depending on the tissue, genetic factors and diets. In SAT, *FABP4* mRNA expression was higher in Alentejano pigs when compared with crossbred pigs and in crossbred pigs fed RPD not adjusted for lysine relative to the control diet. The FABP4 is responsible for the transport of fatty acids in adipocytes and its content is associated with backfat thickness (Michal *et al.*, 2006) and IMF content (Damon *et al.*, 2006).

In this experiment, the most important finding was that the increase of IMF content promoted by RPDs was very likely due to the lysine limitation in the diet. Nevertheless, reducing dietary lysine affects negatively some productive traits, such as G:F and ADG (Teye *et al*., 2006). Amino acids are required for all phases of growing pigs, mainly the essential amino acids, such as lysine. Genotype (protein accretion rate potential) is the most important factor affecting amino acid requirements, but health and environment also have impact. If amino acid intake is inadequate to maximize protein accretion rate, pigs grow slowly and produce fatter carcasses but have more marbling (Pettigrew & Esnaola, 2001). It is well known that lysine is the first limiting amino acid in pig diets because the cereal feedstuffs have low lysine content. The RPDs in this study affected negatively the productive traits in crossbred pigs because lysine requirements are higher than the lysine content available in these diets. Contrarily, Alentejano purebred animals were not affected by RPD, which was very likely due the lysine dietary level was not limiting for this pig breed. In fact Iberian pigs (similar to the Alentejano breed) have a low capacity for lean tissue deposition with maximum average protein deposition rates of 71.0 g/day in pigs with 50 and 100 kg body weight (Barea *et al.*, 2007; García-Valverde *et al.*, 2008).

6.1.2. Arginine and leucine effects on the crossbred pigs

In the second experiment, a different lean pig genetic line was used. This commercial crossbred was a result the four genetic lines, 50% Large White × 50% Landrace gilts mated to 50% Duroc × 50% Pietrain boars, that result a crossbred with 25% Large White × 25% Landrace × 25% Duroc × 25% Pietrain, a commercial porcine genotype with fast growth. This crossbred introduced the Duroc breed, a breed with high IMF content. In this experiment, we investigated the cumulative effect of arginine and leucine on RPDs on meat quality traits and their associated molecular and metabolic mechanisms. The six diets used in this experiment were isoenergetic (14 MJ ME/kg). The protein reduction was 16% to 13%, a decrease of 19% in crude protein. To investigate the arginine effect, 1.0% of arginine was added to three diets and 2.05% of alanine was added to the other three diets, as isonitrogenous controls. Leucine was added in the amount that corresponds to a total final of 2.0% in the diet that was an increase of approximatly 70% of leucine, comparatively to the diets without leucine addition.

The RPDs improve meat quality, mainly by increasing IMF content, confirming our previous results. Dietary supplementation of arginine (Ma *et al*., 2010; Tan *et al*., 2009) and leucine (Cisneros *et al*., 1996; Hyun *et al*., 2003 and 2007) have been reported to increase IMF content when applied to growing-finishing pigs.

Arginine is a semi-essential amino acid and dietary arginine supplementation reduced fat accretion and improved the metabolic profile in Zucker diabetic fatty rats (Fu *et al*., 2005; Wu *et al*., 2007). Some studies with obese rats have demonstrated that arginine administration in fact reduced carcass white fat (Jogben *et al.*, 2006; Wu *et al.*, 2009). Moreover, Ma *et al*. (2010) reported that dietary arginine supplementation reduced stress levels, enhanced whole-body antioxidative function and improved meat quality of finishing pigs, increasing IMF content. Also, Tan *et al*. (2009) concluded that arginine treatment increased muscle protein and fat content, as well as muscle pH at 45 min post-mortem, while reducing lactate content and improving carcass quality in growing-finishing pigs. Tan *et al.* (2009) reported that dietary supplementation with arginine reduced white adipose tissue mass, while improved metabolic profile in the whole body in pigs (He *et al.*, 2009). Also, others studies observed that arginine supplementation increase IMF content in pigs, that indicate enhanced lipogenesis in skeletal muscle (Tan *et al.*, 2009; Ma *et al.*, 2010). These results indicated that arginine treatment has a differential effect in white adipose tissue and skeletal muscle, possibly due to tissue-specific regulation of the expression of lipogenic and lipolytic enzymes by this amino acid (Tan *et al.*, 2011). Thus, our expectations were that arginine supplementation would increase the IMF. However, in our experiment, the arginine addition did not increase IMF content. Likewise, other authors found that arginine supplementation did not increase the IMF content in pork (Go *et al*., 2012). In fact, IMF did not increase with

arginine supplementation in the same amount as in the other studies (1%), perhaps due to the fact that genotypes used are genetically different and their metabolic response was different, which is however, not clear.

In our second experiment, only RPDs resulted in increased in IMF content (about 45%) and this increase was accompanied by the up-regulation of *SCD*, which confirms the mechanism suggested in the first experiment. However, RPD affected negatively the productive traits, such as ADFI, G:F and ADG. The decrease of G:F is probably due to increase the ADFI with RPDs, because the animals need to eat more to compensate their requirements. The ADG decreased in pigs fed RPDs, quite possibly due to the limiting lysine levels in this diet.

Few studies suggested that IMF, or marbling levels, of pork can be increased by feeding high levels of leucine to finishing pigs. However, high dietary leucine may be deleterious, affecting negatively growth performance (Hyun *et al*., 2003). In our study, leucine did not increase IMF content neither affected the growth performance, maybe due to the genetic line of pigs used, which was different from that used in the previous studies. Nevertheless, the sensory traits were slightly affected by both leucine and arginine supplementation. Arginine induced an off-flavour in meat clearly detected by the sensory panel. However, arginine supplementation did not modify the free arginine content in muscle, assessed 17-19 h after feed intake. Thus, the cause for the off-flavour induced by dietary arginine is not clear. Maybe some degradation products of arginine accumulate in the muscle or released during pork thermal processing, can contribute to the detected off-flavour. Also, arginine addition increased juiciness in normal protein diet. We did not find any study about the effect of arginine supplementation on sensory traits on growing pigs. Thus, our study reports for the first time a clear effect of dietary arginine supplementation on sensory quality of pork. An increase of tenderness and overall acceptability was found with arginine and leucine on RPD.

In pigs, the fatty acid composition of muscle and SAT is highly dependent of fatty acid composition of the diet. In spite of that, intense *de novo* lipogenesis occur in growing pig modulating the dietary influence. Modulation of lipogenic metabolism gene expression and consequent effects in fatty acid composition of body tissues has been achieved using amino acids dietary supplementation. Our results showed that arginine supplementation increased total fat in adipose tissue but not in muscle. It is possible that increasing total fat in adipose tissue is associated to an up-regulation of *SCD*. In muscle, although arginine affected the expression of some genes involved in lipid biosynthesis, no effects were detectable in both IMF and fatty acid composition. However, the LC-PUFA decrease with arginine supplementation and this probably is related to the down-regulation of delta 5 desaturase (FADS1). This enzyme is required for the synthesis of LC-PUFAs (Nakamura & Nara, 2004). Regarding fatty acid composition, Tan *et al.* (2011) found that arginine supplementation increased the proportion of oleic acid in skeletal muscle. However, in our work this fact was

not observed nor in muscle neither in SAT. This result which is different from another previous study can be explained by the distinct pig genotypes used.

Leucine is an essential and ketogenic amino acid, the carbon skeleton of which is converted to acetyl-CoA and acetoacetate in muscle tissue, and those intermediated can be used to synthesize fatty acids (Hyun *et al*., 2003). In our work, the leucine supplementation did not affect IMF content but had a considerable effect on fatty acid composition in muscle increasing MUFA and decrease PUFA. This fact could be associated to the up-regulation of key lipogenic enzymes, such as *FASN* and *SCD*. In SAT, leucine had no effect on fatty acid composition, in spite of the up-regulation of some genes of lipid metabolism, mainly *SCD*.

6.1.3. Major outcomes of this research

These two experiments used three different genetic lines of pigs, one fatty pig breed (Alentejana purebred) and two commercial lean pig crossbreds. Alentajena purebred is a Portuguese autochthonous breed and is characterized to deposit high fat levels and has a slow growth. We chose this breed with the objective to investigate if the muscle lipogenic effect of RPD previously described on commercial crossbred pigs was also present in a fatty breed. The crossbred used in the first experiment was a lean pig (50% Large White × 25% Landrace × 25% Pietrain). In the second experiment, we used also a commercial crossbred pig but different from that used in the first experiment. The crossbred pigs of the second experiment were 25% Large White × 25% Landrace × 25% Duroc × 25% Pietrain. This crossbred was 25% less of Large White line and was 25% of Duroc line, when compared with the crossbred used in the first experiment. We chose this crossbred with 25% of Duroc genetic line because this crossbred was expected to have a strong propensity for high levels of IMF content (Cánovas *et al.*, 2010). In spite of this, our Duroc crossbred pigs showed lower IMF than the crossbred pig genotype used in the first experiment (1.43% *vs*. 2.68%) when fed control protein diet. One possible explanation is that Duroc line shows high levels of IMF content only when it is in pure breed or higher percentage of Duroc genes. Meinert, Christiansen, Kristensen, Bjergegaard & Asslyng (2008), in a crossbred with 50% Duroc × 25% Landrace × 25% Large White obtained 1.7% IMF content, which is in accordance with our results. Cánovas *et al.* (2010) performed a breeding program and got a lean and a fatty pig line for Duroc breed, one with high (7.46%) and the other with low (3.58%) IMF content.

An overview of the main results obtained in the two experiments is presented in Table 6.1.

Table 6.1 – Overall findings of RPDs and amino acid supplementation effects in the experiments 1 and 2. The results are compared with the respective control.

Experiment 1				
	Alentejano		**Crossbred**	
	RPDL	**RPD**	**RPDL**	**RPD**
Productive/carcass traits	↑ ADFI	↑ ADFI	↑ ADFI	↑ ADFI
	↓ G:F	↓ G:F	↓ G:F	↓ G:F
	= ADG	= ADG	= ADG	↓ ADG
	=P2	= P2	= P2	= P2
Muscle	= IMF	= IMF	= IMF	↑ IMF
	= 18:1*c*9	= 18:1*c*9	= 18:1*c*9	= 18:1*c*9
	↑ SFA	↑ SFA	↑ SFA	↑ SFA
	↓ PUFA	↓ PUFA	↓ PUFA	↓ PUFA
	= *SCD*	= *SCD*	= *SCD*	↑ *SCD*
Pork	= Juiciness	= Juiciness	= Juiciness	↑ Juiciness
Subcutaneous adipose tissue	↑ TFA	↑ TFA	↑ TFA	↑ TFA
	↓ PUFA	↓ PUFA	↓ PUFA	↓ PUFA
		↓ *ACACA*		↑ *FABP4*
		↑ *LPL*		

Experiment 2			
	Arg	**RPD**	**Leu on RPD**
Productive/carcass traits	= ADFI	↑ ADFI	= ADFI
	= G:F	↓ G:F	= G:F
	= ADG	↓ ADG	= ADG
	= loin weight	↓ loin weight	= loin weight
	= P2 backfat tickness	↑ P2 backfat tickness	=P2 backfat tickness
Muscle	= IMF	↑ IMF	= IMF
	= 18:1*c*9	= 18:1*c*9	↑ 18:1*c*9
	= SFA	↓ SFA	= SFA
	= MUFA	= MUFA	↑ MUFA
	↓ *n-3* PUFA	↓ *n-3* PUFA	↓ PUFA
	↓ *FABP4*	= *FABP4*	↓ *FABP4*
	↑ *FASN*	= *FASN*	↑ *FASN*
	= *LPL*	↑ *LPL*	↓ *LPL*
	↓ *MLXIPL*	= *MLXIPL*	↓ *MLXIPL*
	↓ *PPARγ*	= *PPARγ*	↓ *PPARα*
	= *SCD*	↑ *SCD*	↗ *SCD*
	↑ *SREBP1*		
Pork	Off-flavour	↑ Juiciness	↑ Flavour
	↑ Juiciness		

Subcutaneous adipose tissue	↑ Total fat	↑ Total fat	= Total fat
	= 18:1*c*9	↑ 18:1*c*9	= 18:1*c*9
	= MUFA	↑ MUFA	= MUFA
	= *n-3* PUFA	↓ *n-3* PUFA	= *n-3* PUFA
	= *CRAT*	= *CRAT*	↑ *ACACA*
	= *FADS1*	= *FADS1*	↓ *CEBPα*
	= *FADS2*	= *FADS2*	↓ *CRAT*
	↑ *FASN*	↑ *FASN*	↑ *FADS1*
	= *LPL*	↑ *LPL*	↑ *FADS2*
	↑ *SCD*	= *SCD*	↓ *FASN*
			= *LPL*
			↑ *SCD*

Cumulative effects

	Arg+RPD+ Leu	**Arg+RPD**	**RPD+Leu**
Muscle	↑ *de novo* synthesis	n.d.	↑ MUFA
			↓ PUFA
Pork	↑ Tenderness	n.d.	n.d.
	↑ Overall acceptability		
Subcutaneous adipose tissue	↑ *de novo* synthesis	↑ Total fat	↑ *de novo* synthesis
		↑ *de novo* synthesis	↓ β-oxidation

RPDL, Reduced protein diet with lysine adjustment; RPD, reduced protein diet without lysine adjustment; ADFI, average daily feed intake; G:F, gain:feed; ADG, average daily gain; P2, P2 backfat thickness; IMF, intramuscular fat; SFA, saturated fatty acids (% total fatty acids); MUFA, monounsaturated fatty acids (% total fatty acids); PUFA, polyunsaturated fatty acids (% total fatty acids); TFA, total fatty acids; ACACA, acetyl-CoA carboxylase; CRAT, Carnitine O-acetyltransferase; FABP4, fatty acid binding protein 4; FASN, fatty acid synthase; LPL, lipoprotein lipase; MLXIPL, interacting protein-like; PPARα, peroxisome proliferator-activated receptor alpha; PPARγ, peroxisome proliferator-activated receptor gamma; FADS1, fatty acid desaturase 1; FADS2, fatty acid desaturase 2; SCD, steroyl-CoA desaturase; SREBP1, Sterol regulatory element binding protein 1↑ increased; ↓ decreased; ↑ tendency to increase; = not changed; n.d., not detected.

Overall we tested nine diets in the two pig experiments. The control diets contained 17.5% and 16% of crude protein (as fed basis) in the first and second experiment, respectively. However, in the two experiments, the RPDs contained approximately 13% of crude protein, which corresponds to 26% and 19% less crude protein for the first and second experiment, respectively. In both experiments, the approximately less 40% of lysine in RPD compared with control lead to similar increases of about 43% on IMF in the crossbred pigs. This suggests that the main determinant of muscular lipogenic response induced by RPD is, in fact, the level of lysine restriction. Probably the mechanism associated to this effect leads to the increase of the *SCD* expression in muscle. Therefore, in the first experiment the RPD up-regulated the *SCD* expression and *PPARγ* showed a similar trend in muscle, but the 18:1*c*9 proportion did not confirm that the RPD suggests an enhancement of the SCD expression. However, in the second experiment the *SCD* expression also tends to increase with RPD

and this was followed by the increase of the IMF content. In addition, total fatty acids and total fat of SAT in the two experiments and backfat tickeness on the second experiment also increased with RPDs. For this reason, the RPDs did not modify the fat partitioning as expected, according to the others authors that reported that RPDs increase the IMF in pig meat with a smaller effect on the amount of SAT (Doran *et al.*, 2006).

In both experiments, RPDs increased IMF content in crossbred pigs (lean pigs), and this increment was reflected on meat quality, increasing the juiciness in both experiments and the overall acceptability in first experiment of crossbred pigs. In Alentejano purebred pigs, these diets did not change the sensory traits, probably because the IMF was already quite high in all treatments and no further increase on IMF was observed. In both experiments, the RPDs affected negatively the productive traits (ADFI, G:F and ADG) in crossbred pigs, which compromise their practical application. Thus, more research is necessary to try to retain the positive muscular lipogenic response and avoid the negative productive effects. The RPD did not affect the Alentejana breed pigs perhaps due to the fact that lysine did not limit protein deposition comparatively to the crossbred pigs. The RPD only increased backfat thickness on crossbred pigs with 25% Duroc line. There was a different response of the Alentejano purebred and the two crossbred pigs studied under the RPD in the two experiments that could be due the genotype-specific expression of key lipogenic enzymes. The *SCD* expression only increased in crossbred pigs fed with RPD in *longissimus lumborum* muscle, contrarily to the Alentejano breed pigs. However, in the Alentejano breed, SAT had higher turnover of lipid metabolism genes with higher expression levels of *ACACA*, *FABP4*, *FASN*, *PPARα*, and *SCD*, when compared with the SAT in crossbred pigs.

In addition, RPDs have been used to decrease the environmental impact, through decreasing of ammonia emission (Dourmad & Jondreville, 2007; Galassi, Colombine, Malagutti, Crovetto & Rapetti, 2010). In pig production, it has been demonstrated that nitrogen excretion in growing-finishing pigs can be reduced by about 10% for each 1% reduction of dietary crude protein (Le Bellego, van Milgen & Noblet, 2001), due to the reduced deamination of excess amino acids and the subsequent synthesis and excretion of urea (Fuller, Reeds, Cadenhead, Seve & Preston, 1987).

Arginine and leucine dietary addition did not increase IMF content as expected, but had an important effect on gene expression levels of lipid metabolism in *longissimus lumborum* muscle and SAT. In addition, arginine supplementation increased total fat in SAT. A decrease of *FABP4* and some transcription factors and an increase of *FASN* expression in muscle were observed with leucine addition. Also, leucine addition increased MUFA and decreased PUFA proportions and there was a tendency to increase the *SCD* (P=0.09) with leucine addition. Although leucine addition did not increased total fat in SAT, increased its *SCD* expression. Arginine supplementation introduced an off-flavour on pork and increased

juiciness on NPD, while leucine supplementation increased the flavour on pork and had a cumulative effect with RPD in the juiciness in control group. Also, overall acceptability increased with the cumulative effect of RPD, arginine and leucine supplementation. Arginine had an effect on fat partitioning when increased total fat in SAT and backfat thickness, but did not increased IMF content. However, this effect was on a contrary direction of what we intend, because our goal was to improve fat partitioning increasing the IMF content without affect SAT.

In both experiments, there were distinct effects on fatty acid composition and gene expression of all diets in muscle and SAT. One of the objectives of this work was to increase IMF content, without increase of total fat in SAT and backfat thickness. However, although these strategies increased IMF content, also increased total fat of SAT and backfat thickness. Different diets had different effects on fatty acid composition in muscle and SAT, *i.e*., on fat partitioning. However, in the first experiment, RPD decreased PUFA proportions in muscle and SAT, but increased SFA proportions in muscle. Contrarily, in the second experiment, RPD decreased SFA in muscle and increased MUFA proportions in SAT. However, dietary leucine addition increased MUFA and decreased PUFA in muscle and *SCD* expression increased in SAT and tended to increase in muscle. These results are consistent to the others studies (Doran *et al.*, 2006).

The genes linked to lipid metabolism in muscle and SAT showed different expression levels, with *FABP4*, *FASN*, *LPL*, *PPARγ* and *SCD* more expressed in SAT than in muscle. This fact can be explained because these genes are markers of adipocyte development and also because SAT is the main tissue where occurs *de novo* synthesis of fatty acids in pigs. Finally, the gene expression was more affected by diets in SAT, than in muscle, in both experiments.

6.2. Conclusions

The results presented here indicate that the use of RPD without lysine adjustment during the growing-finishing phase of crossbred pigs increase IMF content in pork and improve its sensory traits, mainly juiciness. The effect of RPD is likely due to reduction of lysine intake to levels below that required for protein deposition in the diet. The possible mechanism associate with this increase IMF seems to be the up-regulation of *SCD* and, consequently, the increase of *de novo* fatty acid synthesis. However, RPD without lysine adjustment also affect negatively some productive traits. RPD do not induce any of these effects on Alentejano purebred (fatty pig genotype), probably due to the lower protein accretion rate and thus lower lysine requirements.

In addition, the data indicate that the RPDs with and without lysine adjustment increase SFA and decrease PUFA in muscle. Moreover, in SAT RPDs increased total fatty acids, total fat and MUFA and decreased PUFA proportions. The fatty Alentejano purebred had a more intense lipid metabolism, with higher fatty acid deposition in muscle and SAT relative to lean crossbred pigs.

Dietary arginine supplementation during the growing-finishing phase of commercial crossbred pigs does not seem to increase IMF content, but increases juiciness and introduces an off-flavour in pork. In addition, the data indicate that arginine supplementation do not affect growth performance. In muscle, arginine seems to have a marked effect on genetic expression associated to lipid metabolism. Arginine also seems to increase total fat in SAT, which is very likely due to the up-regulation of *FASN* and *SCD*.

Dietary leucine addition does not seem to increase IMF content, but in muscle may increase MUFA and decrease PUFA and tends to up-regulate the *SCD* expression, which could be the mechanism responsible for the increased MUFA. Leucine addition in diet seems to affect sensory attributes and increase flavour of pork. In SAT, leucine addition up-regulated *SCD*, *FADS1* and *FADS2*, but this increase does not seem to reflect PUFA composition.

Regarding the cumulative effects obtained with arginine, RPD and leucine, the results suggest an increase of *de novo* lipid synthesis in muscle and SAT, tenderness and overall acceptability of sensory traits of pork. Moreover, RPD supplemented with arginine seems to increase total fat in SAT through the increase of *de novo* fatty acid synthesis. Also, leucine addition to RPD seems to increase *de novo* fatty acid synthesis and decreases β-oxidation in SAT.

Overall, the results obtained in the two experiments indicate that lipogenesis are regulated differentially in the *longissimus lumborum* muscle and SAT of pigs, and that this modulation is specific of the genotype in study. These specificities enable perspectives of fat partitioning and differential effects in different genotypes. The best strategy to improve fat partitioning explored in this thesis was the RPD without lysine adjustment.

6.3. Implications and future perspectives

The findings of this investigation may contribute to the development and improvement of genotype-specific feeding strategies to satisfy the consumer requirement and to enhance the competitiveness of the meat industry with higher quality meat and lower production costs.

The RPDs have an important effect on fat partitioning modification with increased IMF content. This increased IMF may improve pork quality traits, including sensory traits. However, it will be important to explore better these strategies with more pig genotypes, and to test the effect on productive traits and growth performance in pigs. In addition, since RPDs are cheaper than the diets with normal protein, they may be profitable to pig prodution.

Furthermore, RPDs are also important due to their lower environmental impact because these feeding strategies reduce nitrogen excretion and ammonia emission.

It seems clear that fat deposition and partitioning in pigs can be modulated by animal nutrition. However, more research efforts will be needed to prevent negative impact on quantitative production traits and on the regulation of fat deposition in the different carcass depots.

The influence of RPD on gene expression associated with lipid metabolism should be more explored, with other complementary techniques, such as western-blotting and determination of desaturases activity, to confirm that RPDs increase the SCD activity, others desaturases and modification of the genetic mechanisms.

Regarding the possibility of improving fat partitioning with arginine and leucine supplemented diets and an increase of IMF content and consequently to improve sensory traits, our results are not conclusive. However, in the literature, some studies have demonstrated that arginine or leucine addition in diets on commercial growing-finishing pigs increase IMF content. Thus, it is necessary to continue to explore this feeding strategy, by using different proportions of amino acids and other genetic lines of pigs. Also, it is important to clarify the off-flavour found in pork with arginine supplementation of pig diets.

Dietary arginine supplementation increased total fat of SAT and changed the expression of many genes associated to the lipid metabolism in *longissimus lumborum* muscle and SAT. Therefore, it is necessary to further explore the effect of arginine on muscle and SAT, mainly the influence on fat deposition.

Leucine supplementation of RPDs tended to increase *SCD* mRNA expression levels, which was in accordance with the increase of MUFA content in *longissimus lumborum* muscle. This result is an important finding, as suggests that dietary leucine supplementation has an additional effect on RPDs. However, in SAT, the *SCD* expression increased with leucine supplementation but did not increase MUFA content. These results should be further explored with additional leucine concentrations and other pig genotypes.

Finally, a major goal of the studies of feeding strategies (RPDs and amino acids supplementation) should be to continue to further elucidate the mechanisms associated with the regulation of lipid metabolism *in vitro* and *in vivo* as well as fat partitioning in the body.

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